Characterization of BACE2 as a Modulator of Vascular Smooth Muscle Cell Function and Atherosclerosis

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ABBREVIATIONS

Aβ	Amyloid-β
ADAM	A disintegrin and metalloproteases
Akt/PKB	Protein kinase B
AP-1	Activator protein-1
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BACE1	β-secretase cleavage enzyme 1
BACE2	β-site APP-cleaving enzyme 2
bFGF	Basic fibroblast growth factor
BP	Blood pressure
Cox2	Cyclooxygenase 2
c-jun	Jun oncogene
CO	Cardiac output
CREB	cAMP response element-binding protein
CTF	C-terminal fragment
EGF	Epidermal growth factor
ERK1/2	Extracellular signal-regulated kinases
GH	Growth hormone
GSK3	Glycogen synthase kinase 3 α/β
GTT	Glucose tolerance test
HDL	High density lipoprotein
HGF	Hepatocyte growth factor
ICAM-1	Intracellular adhesion molecule-1
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor-1
IκBα	IκB kinase α
IL-1	Interleukin-1
IL-1R1	IL-1 receptor type I
IL-1R2	IL-1 receptor type II
IL-6	Interleukin-6
IRS-1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinases

LDL	Low density lipoprotein
LRP1	Low-density lipoprotein receptor related protein
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein 1
MIF	Macrophage migration inhibitory factor
M-CSF	Macrophage colony-stimulating factor
NF-κB	Nuclear factor κB
p38	P38 mitogen-activated protein kinase
PDGF	Platelet derived growth factor
PI3K	Phosphoinositide-3 kinase
p70S6 kinase	70 kDa ribosomal S6 kinase
PVR	Peripheral vascular resistance
sAPP	Soluble amyloid precursor protein
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor β
TGN	Trans-Golgi network
TNF-α	Tumor necrosis factor-α
VCAM	Vascular adhesion molecule
VSMCs	Vascular smooth muscle cells

SUMMARY

Atherosclerosis is a chronic inflammatory disease of the vascular wall, characterized by the proliferation and accumulation of vascular smooth muscle cells (VSMCs) in the intimal layer of the wall. Previous studies of this laboratory have identified BACE2 (β-site APP-cleaving enzyme 2) as a gene expressed at a higher level in atherosclerosis-protected arteries in experimental atherosclerosis. The aspartyl protease BACE2 is an alternative α secretase which mediates ectodomain shedding of amyloid-β precursor protein (APP) and other transmembrane proteins. BACE2 shares high homology with BACE1 at amino acid level. The β-secretase BACE1 is predominantly expressed in neuronal cells, and is responsible of shedding APP resulting in the generation of the neurotoxic amyloid-β (Aβ) in Alzheimer's disease. BACE2 is expressed at low level in the brain but is found to be highly expressed in vascularized tissues. Moreover, BACE2 preferentially cleaves APP at a different site, resulting in the formation of a non-amylogenic product. The similarity between Alzheimer's disease and atherosclerosis and the high expression of the secretase in atherosclerosis-resistant arteries, led to speculate a role of BACE2 in the regulation of atherosclerosis. The aim of the present dissertation was to characterize the expression, regulation and function of BACE2 in VSMCs *in vitro* and the effect of BACE2 on atherosclerosis *in vivo*.

This study demonstrates for the first time that BACE2 is expressed in human VSMCs. Using immunofluorescence analysis BACE2 was localized to caveolae, trans-Golgi network and endosomes. Furthermore, upon stimulation of cell growth, a rapid proteasome-dependent degradation of BACE2 was observed. Overexpression of BACE2 in VSMCs resulted in inhibition of cell proliferation, and was accompanied with downregulation of c-jun- and IκBα-dependent pro-inflammatory signaling pathways. On the other hand, downregulation of BACE2 using specific siRNA enhanced proliferation of these cells. To study the potential role of BACE2 *in vivo*, mice systemically overexpressing human BACE2 were generated and than crossed with $ApoE^{-/-}$ mice. The $ApoE^{-/-}$ BACE2-Tg mice had reduced atherosclerosis compared to $ApoE^{-/-}$ control mice. Furthermore, blood plasma concentrations of the pro-inflammatory cytokine IL-1β, cholesterol and lipids were also reduced. These results suggest a protective role of BACE2 in atherosclerosis, by attenuating proliferation of VSMCs, inflammation, and plasma levels of cholesterol and lipids. Thus, specific induction and activation of BACE2 could provide a novel therapeutic approach to decrease progression of atherosclerosis.

ZUSAMMENFASSUNG

Atherosklerose ist eine entzündliche, chronische Gefäßkrankheit, die sich durch Vermehrung und Ablagerung von vaskulären, glatten Muskelzellen in der inneren Schicht der Arterienwand auszeichnet. In früheren Studien dieses Labors wurde gezeigt, dass BACE2 (β-site APP-cleaving enzyme 2) stärker exprimiert wird in Arterien, die gegen Atherosklerose geschützt sind. Die Aspartylprotease BACE2 (β-site APP-cleaving enzyme 2) ist für die enzymatische Spaltung der extrazellulären Domäne des Beta-Amyloid Vorläufer Proteins (APP) oder anderer transmembraner Proteine verantwortlich. BACE2 weist eine grosse Ähnlichkeit in der Aminosäuresequenz zu BACE1 auf. BACE1 wird hauptsächlich in Neuronen exprimiert und vermittelt die enzymatische Spaltung des APP, welches zur Bildung des neurotoxischen Beta-Amyloid Peptids führt. Das Beta-Amyloid spielt eine entscheidende Rolle in der Entstehung von Morbus Alzheimer, einer präsenilen Demenz. BACE2 wird stark in vaskulärem Gewebe exprimiert, wohingegen kaum im Gehirn. Außerdem schneidet BACE2 vorzugsweise APP an einer anderen Erkennungsequenz, welches zur Entstehung eines nicht amylogenen Produktes führt.

Die Homologie zwischen Morbus Alzheimer und Atherosklerose und die erhöhte Expression von BACE2 in von Atherosklerose geschütztem vaskulärem Gewebe, legen die Vermutung nahe, dass BACE2 eine Rolle in der Regulation von Atherosklerose spielen könnte.

Das Ziel der vorliegenden Dissertation war die Charakterisierung der Expression, Lokalisation, Regulation und Funktion der Sekretase BACE2 in vaskulären glatten Muskelzellen *in vitro*. Zusätzlich wurde die Funktion von BACE2 in einem Modell für Atherosklerose, der $ApoE^{-/-}$ defizienten Maus, *in vivo* analysiert.

In der vorliegenden Arbeit wird zum ersten Mal gezeigt, dass BACE2 in menschlichen vaskulären glatten Muskelzellen exprimiert wird. Mit Hilfe der Immunofluoreszenzanalyse konnte bestimmt werden, dass BACE2 sowohl in den Caveolae, im Trans-Golgi Netzwerk und in den Endosomen vaskulärer glatter Muskelzellen lokalisiert ist. Außerdem konnte, nach Stimulation mit Wachstumsfaktoren, ein schneller Proteasom-abhängiger Abbau des Proteins BACE2 festgestellt werden. Die Überexpression von BACE2 in vaskulären glatten Muskelzellen führte zur Hemmung der Zellvermehrung. Zusätzlich führte dies zu einer Herunterregulierung von c-jun und IκBα-abhängigen entzündungsfördernden Signalwegen. Die Herunterregulierung der BACE2 Genexpression durch eine siRNA Transfektion resultierte in einer erhöhten Zellvermehrung desselben Zelltyps.

Um die Funktion von BACE2 *in vivo* zu untersuchen, wurden ApoE$^{-/-}$ BACE2 transgene Mäuse (BACE2-Tg) generiert und mit ApoE-defizienten Mäusen gekreuzt. In den ApoE$^{-/-}$ BACE2-Tg Mäusen konnte, im Vergleich zu den ApoE$^{-/-}$ Kontrollmäusen, eine Reduktion der atherosklerotischen Plaques festgestellt werden. Darüber hinaus war die Konzentration von entzündungsfördernden Zytokinen wie auch die Konzentration von Cholesterin und Fette im Blutplasma verringert. Die vorliegenden Ergebnisse deuten darauf hin, dass BACE2 anti-proliferative und anti-inflammatorische Eigenschaften in Bezug auf Atherosklerose aufweist. Spezifische Induktion und Aktivierung von BACE2 könnte ein neuer therapeutischer Ansatz zur Behandlung atherosklerotischer Erkrankungen sein.

1. INTRODUCTION

1.1 The Cardiovascular System

Heart, blood vessels and blood constitute the cardiovascular system. The human cardiovascular system consists of two blood circulation loops as schematically summarized in **Figure 1**.

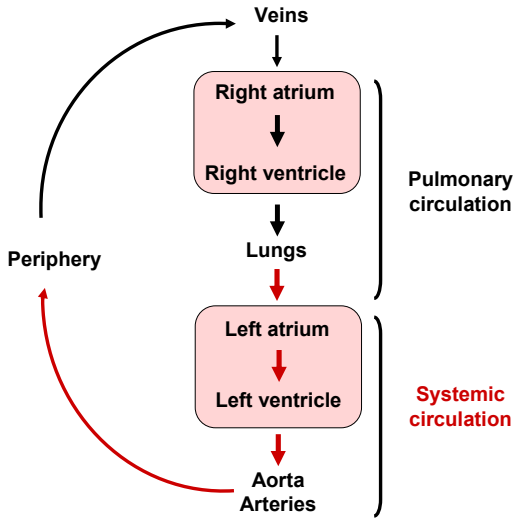


Figure 1: The human cardiovascular system. The human cardiovascular system consists of a pulmonary and a systemic circulation. The pulmonary circulation is a loop between the heart and the lungs and is important for the oxygenation of the blood. The systemic circulation provides the oxygenized blood (represented by the red arrows) to the periphery of the body. Red areas represent the heart.

The human heart is one of the most important organs and is composed of four chambers: two muscular atria and two ventricles. The blood flows from the right atrium into the right ventricle passing through the tricuspid valve, which allows the blood to flow only in one direction. From this ventricle the blood is then pumped to the lungs (pulmonary circulation) and returns into the left atrium via the pulmonary vein. Through the bicuspid valve, the blood flows into the left ventricle, from which it is driven through the aorta to the periphery of the body (systemic circulation). Between the left ventricle and the aorta, the aortic valve prevents flow back of the blood into the heart [1, 2]. The aorta is anatomically divided into five parts: the ascending aorta, the aortic arch, the descending aorta, the thoracic aorta and the abdominal aorta. The aorta, the largest artery of the body, is the first vessel in which the blood flows

when it is pumped out of the heart to reach all organs and tissues of the body. The blood flows through the arteries, the arterioles, the metarterioles and finally reaches the capillary beds. The capillary system connects the arterial vascular tree to the venous system and is responsible for the exchange of water, gases, nutrients, metabolites and waste between blood and tissues. Through venules, small veins and large veins the blood returns into the heart, where it is again pumped into the pulmonary circulation for reoxygenation [1, 2].

The flow of the blood through the body is mediated by the beating of the heart [1]. However, differences in pressure (pressure gradient) are also important to keep the blood flowing. Blood always flows from high to low pressure [1]. Blood pressure (BP), which is the force produced by the blood flow on the vessel wall, is determined mainly by the cardiac output (CO) and the peripheral vascular resistance (PVR): BP= CO x PVR [3]. The peripheral resistance mainly depends on the blood viscosity, the length of the vessel and the vessel diameter [1].

The cardiovascular system is a complex constellation of blood pressure, blood distribution and transport of several vital elements. In the Western world the cardiovascular system seems to be one of the most vulnerable part of the body [4]. This is shown by the fact that today diseases affecting the cardiovascular system account for 30% of worldwide deaths [4].

1.1.1 Function of the Blood

The function of the blood is to transmit signals (hormones), transport gases (including O_2 and CO_2), nutrients, vitamins, cholesterol, metabolites, and defend the body from microorganisms, viruses, foreign proteins, parasites, fungi and cancer (immune system).

In order to accomplish this role different types of blood cells are involved. Blood cells arise from a pluripotent hematopoietic stem cell, through a process called hematopoiesis. In adults the bone marrow is the major hematopoietic organ [5]. Hematopoietic stem cells can produce either lymphoid progenitor cells or myeloid stem cells. The lymphoid progenitor cells generate B, T and natural killer cells. These cells are mainly involved in the specific adapted immune system. Myeloid stem cells, on the contrary, give rise to white blood cells such as neutrophils, eosinophils, basophils, monocytes and mast cells, which participate to the nonspecific innate immune system. Additionally, myeloid stem cells also produce red blood cells (erythrocytes), which are responsible for the transport of gases and platelets, needed for blood homeostasis [6].

All these cells may produce biologically active molecules, such as cytokines and growth factors, which regulate hematopoiesis and inflammatory responses [6].

1.1.2 Arterial Structure

The anatomy and structure of an artery depends on its function, size and position within the body. Elastic arteries are located close to the heart and are of a large diameter (in humans: 1 to 2.5 cm). This type of artery is composed of elastic tissues and therefore rhythmically expands and recoils helping the blood to move onward. Examples of elastic arteries are the aorta and its branches such as the carotid artery, the subclavia and the renal artery [1, 7].

Muscular arteries are located in the periphery (with exception of the coronary arteries) and are thinner (in humans: 0.3 to 1 cm) and more muscular compared with elastic arteries. The wall of muscular arteries consists of a thick layer of vascular smooth muscle cells (VSMCs), which shows an active role in vasoconstriction (decrease of lumen diameter) and vasodilation (increase of lumen diameter) to regulate blood flow and blood pressure [1, 7].

Histologically, the arterial wall is a well-structured tissue, which consists of three distinct layers: the tunica intima, the tunica media and the tunica adventitia (**Figure 2**) [7]. The thickness of these three layers varies depending on the type of vessel [8-11].

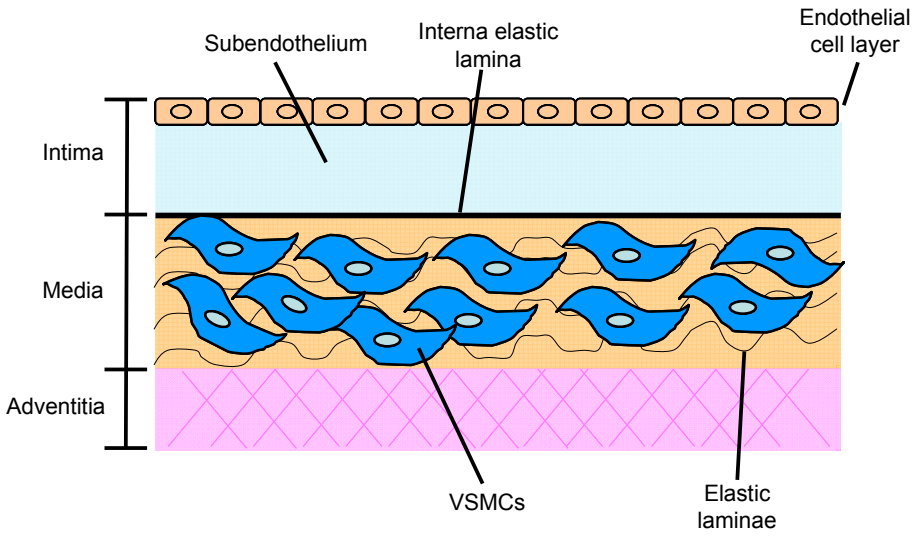


Figure 2: Structure of an artery. An artery consists of three morphologically distinct layers: The tunica intima, the tunica media and the tunica adventitia. The tunica intima consists of a single layer of endothelial cells and a subendothelial space. An interna elastic lamina provides flexibility and separates the intima from the media. The tunica media is composed of VSMCs bounded by elastic laminae. The tunica adventitia, the outer layer, consists mainly of extracellular matrix and collagen fibers.

The tunica intima is the most internal layer of an artery and it is composed of a single layer of endothelial cells, the subendothelium and the internal elastic lamina, which provides flexibility, stability and separates the intima from the media. The subendothelium is composed of proteoglycans and bundles of collagen (primarily of type I and III) and provides mechanical support to the artery. In the subendothelium VSMCs may be present [7, 9].

The media is the middle layer of the arterial wall and consists predominantly of contractile VSMCs bounded by elastic laminae. VSMCs are embedded in collagen and elastin fibers (primarily of type I and III) [7].

The tunica adventitia is the outer layer of an artery and consists of extracellular matrix and collagen fibers (primarily of type I), where fibroblasts, mast cells and perivascular nerves are embedded. The main function of the adventitia is to provide stability and strength to the arterial wall [7]. In the adventitia of big arteries blood vessels are found, called vasa vasorum, to supply nutrients to the artery [12].

1.1.3 Cells of the Arterial Wall

1.1.3.1 Endothelial Cells

Endothelial cells, which are mounted on a basement membrane (basal lamina) are in direct contact with the blood and act as a selectively permeable barrier between the blood and the tissues. The endothelium regulates the exchange of water and solutes, and controls the traffic of macromolecules, such as lipoproteins. Through the expression of adhesion molecules endothelial cells are also responsible of controlling the adhesion, rolling and extravasation of inflammatory cells, such as macrophages and lymphocytes, through the endothelial layer. This is highly important during inflammatory responses, to allow the influx of cells of the immune system from the blood (at the level of the capillaries) into the tissue where infection is taking place [6].

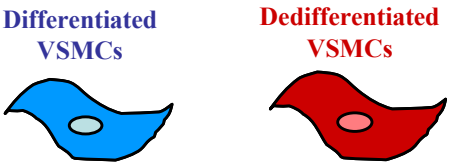
Another important function of endothelial cells is the regulation of vascular tone via different effector molecules, which modulate VSMCs contraction and relaxation [13, 14]. Nitric oxide [15] and prostaglandin I2 [16] for example are released by endothelial cells and act on adjacent VSMCs to induce relaxation, whereas vasoactive factors such as endothelin [17] and prostaglandin F2alpha [18] induces VSMCs contractions.

Endothelial cells are also involved in the regulation of coagulation, participating in blood homeostasis and assuring wound healing. Dysfunction of endothelial cells may lead to diseases such as atherosclerosis [13, 19, 20].

1.1.3.2 Vascular Smooth Muscle Cells

VSMCs are multifunctional cells with remarkable plasticity. In healthy condition VSMCs are located in the medial layer of the arterial wall and show a quiescent phenotype. The main function of VSMCs is the regulation of vascular tone via contraction and relaxation in response to various endothelium- or locally-derived substances [13, 16, 21, 22]. Through contraction and relaxation of VSMCs, arteries change their diameter to regulate blood flow and blood pressure [13]. This process is important for regulating vascular tone [1]. Medial VSMCs express contractile proteins, receptors, ion-channels and signal-transducing molecules which allow these cells to accomplish their specialized contractile function [13, 21]. Among others, the expression of contractile proteins such as smooth muscle 22 alpha, calponin, α- and γ-smooth muscle actins, smooth muscle myosin heavy chain and polyubiquitin are of crucial importance [23].

VSMCs are also capable of profound reversible changes in response to environmental alterations [21]. The most evident example of VSMCs plasticity is observed during vascular development, vascular injury and in vascular diseases like atherosclerosis and hypertension [24]. During vascular development VSMCs play a central role in formation of a new vessel wall. To accomplish this role the cells have to proliferate at a high rate, migrate and produce high amount of extracellular matrix components such as collagen, elastin and proteoglycans. In response to vascular injury and diseases VSMCs show a similar phenotype. However, the most profound switch in phenotype can be observed in atherosclerosis [24, 25]. During Atherosclerosis these cells dedifferentiate and migrate to the intima, where they proliferate at a higher rate and produce huge amounts of extracellular matrix, cytokines and proteases [26] (**Figure 3**).



State	Healthy	Diseased
Arterial localization	Medial	Intimal
Contractile function	Yes	No
Proliferative phenotype	No	Yes
Extracellular matrix production	Low	High
Protease production	High	Low
Cytokine secretion	Low	High

Figure 3: Phenotypic switching of VSMCs during atherogenesis. This figure summarize the different phenotypes and characteristics between a quiescent and differentiated VSMC present in the media (left) and a dedifferentiated highly proliferative intimal VSMC present in atherosclerotic lesions (right).

1.2 Cardiovascular Disease and Atherosclerosis

Diseases causing a detrimental effect to the cardiovascular system are the leading cause of death in Western countries [4]. Currently, every year more than 17 million people die from direct or indirect consequences of cardiovascular diseases worldwide. This represents 30% of total deaths [4].

Cardiovascular diseases include coronary artery disease (leading to heart attack), peripheral artery disease, cerebrovascular disease (leading to ischemic stroke), heart failure, hypertension, rheumatic and congenital heart disease [2, 4, 27]. The most common cardiovascular disease is atherosclerosis [2, 27]. Atherosclerosis is a multifactorial, systemic, progressive, chronic, inflammatory disorder of the arterial wall of medium-size and large arteries, characterized by the accumulation of fatty deposits in the inner layer of the arterial wall (the tunica intima) [19]. Atherosclerosis may cause narrowing and loss of flexibility of the arterial wall and, in some cases, the sudden rupture of an atherosclerotic plaque and subsequent thrombosis may lead to the total occlusion of the lumen [19, 28-30]. Rupture of vulnerable plaques represents the leading cause of myocardial infarction (75%) [31] and of ischemic stroke (90%) [32].

1.2.1 Risk Factors of Atherosclerosis

Several risk factors contribute to the development and progression of atherosclerosis and cardiovascular diseases in general; some can be controlled or treated, others not.

1.2.1.1 Controllable/Treatable Risk Factors

Among risk factors which may be controlled is hypercholesterolemia, which is manifested by an elevated plasma level of low density lipoprotein (LDL) and a reduced level of high density lipoprotein (HDL). This represents the most critical risk factor for the initiation and progression of atherosclerosis [28]. However, severe genetic anomalies affecting the lipoprotein metabolism such as mutations in the LDL receptor gene or in the ApoE gene, are known to induce high level of hypercholesterolemia which may not be sufficiently controlled and treated [33, 34].

Another important risk factor for the development of cardiovascular diseases, which may be easily controlled, is smoking. Smokers have in fact two to four times higher risk for coronary heart diseases compared with nonsmokers [35]. Hypertension [3, 36], dyslipidemia [37], diabetes mellitus [38] and insulin resistance [39] are also known to increase the risk for stroke, heart attack and kidney failure. Additionally, sedentary life style [40] and excessive intake of fat [41] may also negatively modulate the course of the disease. Overweight and obese people have in fact a higher risk for cardiovascular diseases compared to lean persons [42].

Prevention is an important step in order to inhibit atherosclerotic plaque development. A very efficient prevention step is the change of lifestyle by stopping to smoke, dietary interventions or physical activities. Additionally, LDL and blood pressure lowering agents, and anti-thrombotic drugs can also help to reduce the risk for atherosclerosis or even promote its regression [43, 44].

Since the risk factors for atherosclerosis are widely prevalent in our society, the disease has been one of the main health problem worldwide for a long time [4].

1.2.1.2 Uncontrollable Risk Factors

Aging is the most important risk factor which can not be controlled. The early stage of atherosclerosis, which is the formation of so called fatty streak, may already begin during fetal development and during infancy [45]. During aging complex metabolic changes occur contributing to the progression and complexity of the disease [46].

Interestingly, the incidence of cardiovascular diseases is higher in men than in women [47, 48]. Accordingly, estrogen deprivation such as menopause has been shown to be an important risk for cardiovascular diseases [47, 48].

Genetic components also play a major role in the onset and progression of the disease [49]. The risks are often hereditary, and therefore family history may significantly influence its development [28]. The investigation of genes that influence the development of atherosclerosis have been studied by several groups and is still an intensive field of research [49]. Genetic variations such as mutations and polymorphisms may define genetic susceptibility and profoundly affect the onset and severity of the disease [50]. For instance, genetic variants of the lipoprotein lipase gene have been associated with dyslipidemia [51] , a polymorphism in the red cell Na and K transport gene with hypertension [52] and a polymorphism of angiotensin I-converting enzyme gene with hypertension and insulin metabolism [53, 54].

1.2.2 Pathogenesis of Atherosclerosis

The course of human atherosclerotic lesion development has been classified by the American Heart Association on the basis of histological changes in six consecutive lesion types (Type I-VI). A schematically representation is shown in **Figure 4:**

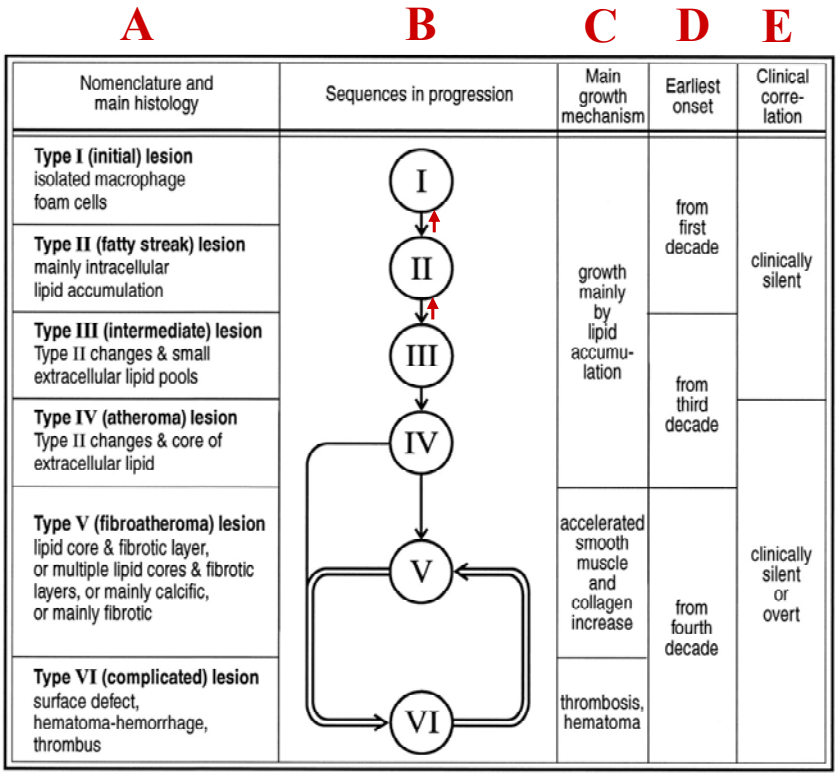


Figure 4: Representation of consecutive changes in development of human atherosclerotic lesions as classified in lesion types I to VI by the American Heart Association.
In Column A, the characteristics of the lesion types are listed. In B, a flow chart showing the sequence of lesion progression is shown. Type II and III lesions are still reversible as indicated by the double-headed arrow. Types V and VI lesions are connected by a loop to depict the increase of lesions thickness when thrombotic events occur. In C, the mechanisms of lesion growth, in E the period of life in which the lesions occur, and in E the clinical correlation are listed. Adapted from [55].

In 2000 Type VII and VIII lesions are added to the list in order to distinguish lesions which develop from Type IV to VI lesions as a result of regression or change of lipids in the atherosclerotic lesion [56]. Type VII lesions are characterized by calcification, whereas type VIII lesions by fibrous tissue changes [56].
In the following the cellular and molecular changes occurring during atherosclerosis development are described and correlated to the histological AHA lesion classification.

1.2.2.1 Endothelial Dysfunction (Lesion type I to III)

The first and crucial event for the initial development of an atherosclerotic lesion is endothelial cell dysfunction [19]. High plasma lipid concentration is considered to be a key factor inducing endothelial dysfunction [19, 26]. Endothelial cell dysfunction is characterized by

functional changes such as abnormal vascular reactivity, an increased permeability of the endothelial layer for LDL, an increased adhesiveness for macrophages and platelets, and production of vasoactive molecules such as cytokines and growth factors [19]. While LDL particles present in the plasma are protected from modification, LDL internalized in the subendothelial space may undergo oxidative modification and become more atherogenic [57]. The accumulation of LDL particles in the arterial wall induces endothelial cells to express high amounts of cell adhesion molecules, chemotactic proteins and growth factors, which favor the adhesion and migration of leukocytes and monocytes into the subendothelium [10, 28] (**Figure 5**). P- and E-selectins, the intracellular adhesion molecule-1 (ICAM-1) and the vascular adhesion molecule (VCAM) are of high importance for adhesion, rolling and extravasation of monocytes and lymphocytes through the vascular wall [58, 59].

Chemotactic molecules such as monocyte chemotactic protein 1 (MCP-1) and growth factors such as macrophage colony-stimulating factor (M-CSF) recruit monocytes into the vessel wall and have a crucial role in stimulating the differentiation of monocytes in macrophages and the proliferation of these cells [10, 28].

1.2.2.2 Fatty Streak Formation (Lesion type II)

Monocytes which enter the arterial wall differentiate into macrophages and engulf LDL particles leading to the formation of foam cells [10] (**Figure 5**). Foam cells are efficient secretory cells that can produce a number of pro-inflammatory cytokines and growth factors amplifying the local inflammatory response in the lesion [60]. Interaction of macrophages, foam cells and activated lymphocytes establish a chronic inflammatory state characterized by the release of high amount of cytokines [28]. Foam cells, macrophages, lymphocytes and accumulated LDL molecules contribute to the formation of a fatty-streak, which is the early stage of an atherosclerotic complex lesion. Fatty-streak provokes the thickening of the arterial wall, which is a typical process in atherosclerosis development [19]. At this stage of the disease, fatty streaks are known to be still reversible [61, 62].

1.2.2.3 Intermediate and Atheroma Lesion Formation (Lesion type III and IV)

As the atherosclerotic process advances lesions become more complex and irreversible [28]. Cytokines and growth factors present in the local environment of the plaque contribute to the phenotypic switching of medial VSMCs [25, 63]. During atherosclerosis VSMCs loose their quiescent and contractile phenotype resulting in dedifferentiation and migration from the

media into the intimal space where the cells proliferate and secrete high amounts of growth factors and cytokines [25, 63] (**Figure 5**). These activated VSMC may take up modified lipoproteins (contributing to foam cell accumulation) and synthesize extracellular matrix proteins resulting in fibrous cap formation [19, 64].

Cytokines expressed by VSMCs promote chronic inflammation and progression of atherosclerotic lesions by activating leukocytes, promoting endothelial dysfunction and proliferation of the cells present in the plaque [65]. The transition from a simple fatty streak to a much more complex lesion which invades the lumen of an artery is characterized by a growing mass of VSMCs and VSMCs-derived extracellular matrix [10]. The higher proliferation rate of VSMCs in the intimal layer and the huge production of extracellular matrix are promoted by cytokines and growth factors secreted by macrophages and lymphocytes [10]. Contractile VSMCs present in the media mainly produce type I and type III fibrillar collagen; on the contrary, intimal VSMCs present in atherosclerotic lesions, mostly secrete proteoglycans and fibronectin. This change in local environment is known to alter the proliferation rate of surrounding cells [66].

Lipid-laden cells (macrophages and VSMCs) together with accumulated lymphocytes, cell debris, VSMCs, and VSMCs-produced extracellular matrix, are responsible for the formation of an advanced atherosclerotic lesion [19] (**Figure 5)**. In addition, this phase of lesion development is characterized by interactions between monocyte/macrophages and T cells. The complex crosstalk of inflammatory and vascular cells is reflected by a broad range of cellular and humoral responses resulting in a chronic inflammatory state within the lesion. Activated T cells within the lesion express Th1 and Th2 cytokines [67, 68], whereas activated macrophages and endothelial cells express MHC class II molecules and numerous inflammatory cytokines, such as TNFα, IL-6, and MCP-1 [28]. Histologically Type IV lesion are usually composed of a lipid-rich core, separated from the vessel lumen by an intact fibrous cap covered by endothelium.

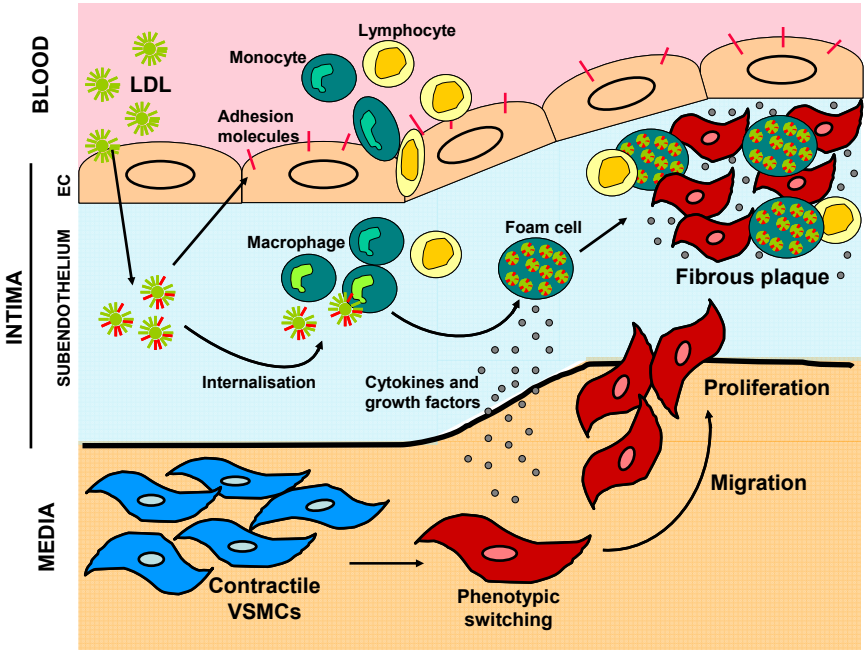


Figure 5: Schematic representation of the different stages involved in atherosclerosis development. Endothelial dysfunction is associated with accumulation of LDL molecules in the subendothelium, where an inflammatory response is triggered, thereby promoting adhesion and migration of lymphocytes and monocytes into the subendothelium. Macrophages, which differentiate from monocytes, accumulate in the arterial wall and internalize LDL, and are thereby transformed in foam cells. These cells produce high amounts of cytokines and growth factors contributing to the phenotypic switching of VSMCs. Dedifferentiated VSMCs migrate from the media to the intima, where they proliferate and secrete high amounts of extracellular matrix. Accumulated VSMCs, VSMCs-derived extracellular matrix together with foam cells, macrophages and lymphocytes, result in the formation of a complex atherosclerotic plaque.

1.2.2.4 Fibroatheroma Lesion (Lesion type V) and Vulnerable Plaque

Type V lesions are characterized by formation of prominent new fibrous connective tissue leading to narrowing of the arteries. The new tissue consists of collagen and proliferating smooth muscle cells. The fibrous cap of an advanced atherosclerotic lesion may become thinner, unstable and brake. This process is promoted on a molecular level by cytokines, such as interferon-γ (IFN-γ) produced by the cells present in the fibrous plaque. Subsequently, macrophages and VSMCs may undergo apoptosis (programmed cell death), resulting in the formation of a necrotic core (lipid-rich core) surrounded by a fibrous cap [29, 56]. Thinning of the fibrous cap is known to be influenced by matrix metalloproteases secreted by macrophages and VSMCs. These enzymes in fact degrade extracellular matrix proteins

rendering the cap unstable [29, 60]. The atherosclerotic lesion is considered as a vulnerable plaque when a big necrotic core and only very thin fibrous cap is remaining [19, 56].

1.2.2.5 Complicated Lesion (Lesion type VI) and Plaque Rupture

Type IV and type V lesions in which the fibrous cap is disrupted (Plaque rupture) resulting in hematoma or hemorrhage have been classified in type VI lesions. In these plaques thrombotic events occurred. After rupture the high thrombotic lipid core is exposed to the blood, causing platelet aggregation and, in most cases, leading to thrombosis, embolization, and vascular occlusion (**Figure 6**). Plaque rupture may result in acute cardiovascular events, such as myocardial infarction and stroke. Fortunately, certain plaques exist which remain stable during the whole life span [29].

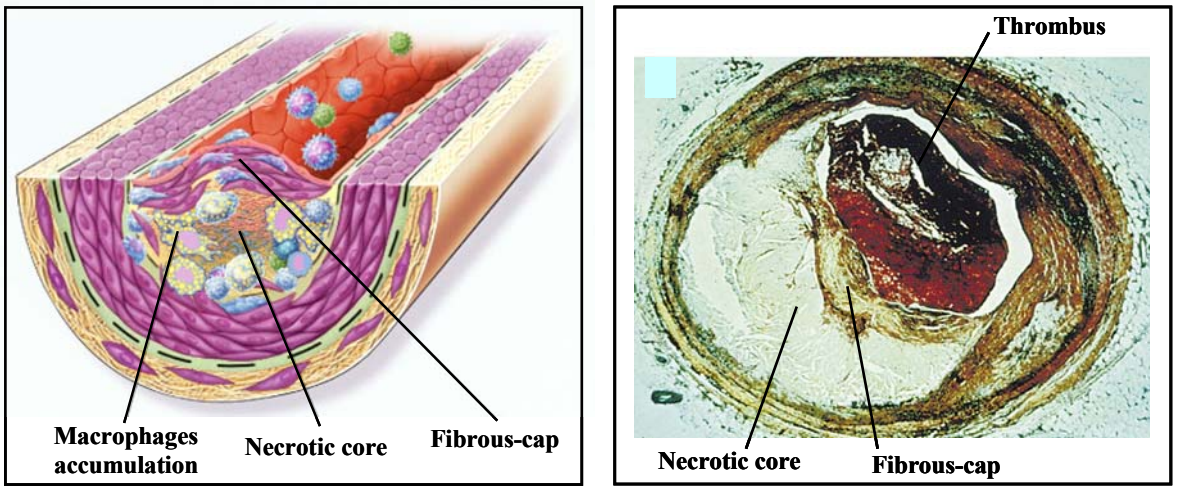


Figure 6: Vulnerable plaque and plaque rupture in atherosclerosis. Left panel: Schematic representation of an advanced atherosclerotic plaque composed by a necrotic core surrounded by a thin fibrous cap. Right panel: Cross section through a coronary artery. Rupture of the fibrous cap may lead to thrombosis, resulting in the occlusion of the arterial lumen.

1.2.3 Inflammation and Atherosclerosis

Atherosclerosis is a chronic, inflammatory disease characterized by the presence of inflammatory cells such as lymphocytes and macrophages in the atherosclerotic lesion [60]. In a healthy condition the endothelium does not permit binding and internalization of these inflammatory cells into the arterial wall. However, after endothelial dysfunction, endothelial cells express adhesion molecules and chemoattractant proteins which promote the recruitment of leukocytes and monocytes into the arterial wall [19, 60]. The inflammatory process is further amplified and regulated by inflammatory cytokines, small signaling molecules produced by white blood cells [6]. During atherogenesis VSMCs and endothelial cells, which in a normal condition do not produce cytokines, also contribute to chronic inflammation of the arterial wall by secreting pro-inflammatory mediators [19, 69].

Cytokines regulate and mediate immune response, inflammation and hematopoiesis and act through binding to specific membrane receptors expressed on neighboring cells (paracrine action) or on the cell of their origin (autocrine action). The activated receptors transmit the information into the nucleus via second messengers (often tyrosine kinases) modulating gene expression profile of the cell [6]. Activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) transcription factors have been shown to be downstream targets of many pro-inflammatory cytokines during atherosclerosis [25, 70-72] and to be associated with phenotype switching and subsequent proliferation of intimal VSMCs [25, 69, 73]. Responses to cytokines include secretion of effector molecules, secretion of other cytokines, increased or decreased expression of membrane proteins (including cytokine receptors), proliferation and migration (**Figure 7**) [6].

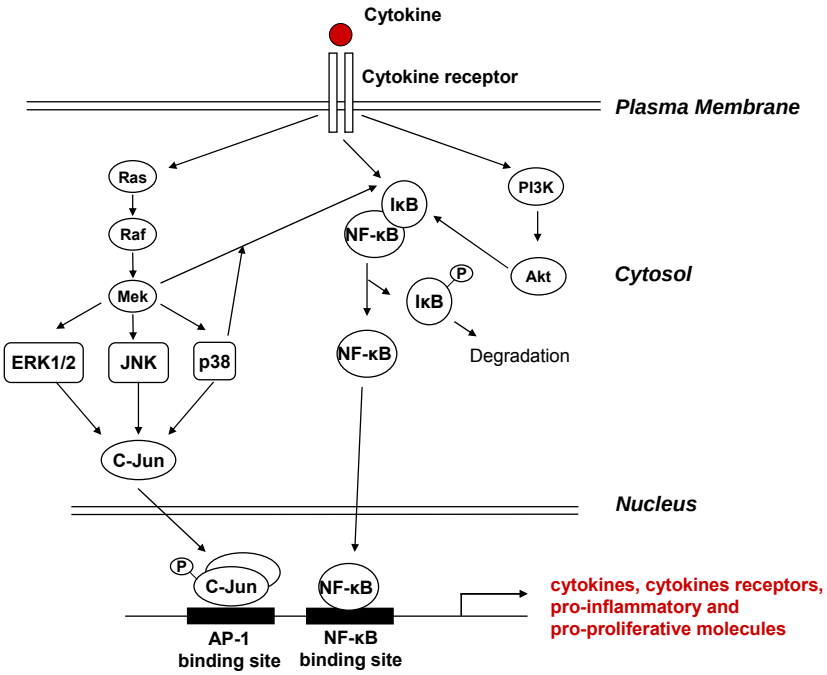


Figure 7: Cytokine signaling pathways. Binding of a cytokine to its receptor results in intracellular activation of different kinase cascades, which regulate the activation of transcription factors such as AP-1 and NF-κB. Activated transcription factors enter the nucleus resulting in the transcription of pro-inflammatory cytokines, cytokine receptors, pro-inflammatory molecules and mitogenic factors. Akt: protein kinase B; AP-1: Activator protein-1; c-jun: Jun oncogene; ERK1/2: Extracellular signal-regulated kinases; IκBα: IκB kinase α; JNK: c-Jun N-terminal kinases; MAPK: Mitogen-activated protein kinases; NF-κB: Nuclear factor κB; p38: P38 mitogen-activated protein kinase; PI3K: Phosphoinositide-3 kinase.

Activation of the dimeric transcription factor AP-1, of which the jun oncogene (c-Jun) is a component, occurs by serine phosphorylation [74]. To activate NF-κB, on the contrary, the degradation of its inhibitor, the IκB kinase α (IκBα), is necessary. In the cytoplasm NF-κB is bound to its inhibitor preventing NF-κB from entering into the nucleus. Serine phosphorylation of IκBα results in ubiquitinilation followed by degradation allowing NF-κB to translocate into the nucleus to activate transcription of target genes [75].

Activation of both AP-1 and NF-κB is mediated by different kinase-dependent signaling pathways. Among others, the phosphoinositide-3 kinase (PI3K)-protein kinase B (Akt)-pathway was shown to be regulated by cytokines leading to the activation of NF-κB [76]. Cytokines also lead to the activation of mitogen-activated protein kinases (MAPK), including c-Jun N-terminal kinases (JNK) [77], extracellular signal-regulated kinases (ERK1/2) [77] and p38 kinase (p38) [78], resulting in the activation of both transcription factors [79-82] (**Figure 7**). Activation of AP-1 and NF-κB transcription factors and their entry into the nucleus result in

the transcription of genes coding for pro-inflammatory and pro-proliferative molecules, which promote and amplify local inflammation in the lesion [82].

During the atherogenic process cytokines are produced in high amounts by different cell types present in the lesion, resulting in a local inflammatory response and VSMC phenotype switching [19]. The inflammatory activation in the vascular wall is not only promoting the initiation of atherosclerotic plaque development, but also contributes to complex and clinical risk such as plaque rupture and consequent thrombotic complications [60].

The most important cytokines produced by intimal VSMCs are platelet derived growth factor (PDGF), transforming growth factor β (TGF-β), macrophage migration inhibitory factor (MIF), IFN-γ, MCP-1 and interleukin-1 (IL-1) [63, 65]. The activated lymphocytes and macrophages which accumulate in the arterial wall are known to release cytokines such as IFN-γ, IL-1, IL-6 and tumor necrosis factor-α (TNF-α) [28]. Finally, foam cells produce cytokines and growth factors such as IL-1, TNF-α, PDGF and basic fibroblast growth factor (bFGF) [60]. Together these cells are responsible for the amplification of the local inflammatory response in the lesion and the progression of the disease. Migration and proliferation of VSMCs in the arterial wall is for instance known to be stimulated by cytokines such as IFN-γ [83], IL-1 [84], IL-6 [85], MIF [86] and TNF-α [87], whereas synthesis of matrix proteins is highly promoted by IFN-γ [88], IL-1 [84] and MIF [86]. IFN-γ also induces VSMCs and macrophages to undergo programmed cell death (apoptosis) [56]. Anti-inflammatory cytokines are also produced, which have the potential of attenuating the immune responses and counter regulate atherosclerosis progression. Examples are IL-10, IL-18 and TGF-β which inhibit VSMCs proliferation and dedifferentiation [89-91].

1.3 Heterogeneity of Atherosclerosis in Humans

Atherosclerosis does not homogenously develop in all arteries of the human body. Generally, atherosclerotic plaques occur in medium- and large-sized arteries and mainly develop in regions of artery bifurcations and curvatures where the blood shows a complex and irregular flow pattern [11]. These regions are susceptible towards development of chronic inflammation and endothelial cells denote an increased permeability to macromolecules such as LDL, which is the crucial event for the initiation of atherosclerosis [10].

Besides the size of an artery and the differences in blood flow, other aspects seem to play an important role. It is in fact well known that within the arterial system, some arteries are more protected from atherosclerosis than others [92]. The internal mammary artery and the radial arteries for examples are highly resistant to the development of atherosclerotic plaques [93-96]. In contrast, coronary arteries, renal arteries, carotid arteries and the aorta exhibit on the contrary a particular predisposition to atherosclerosis development [97].

This anatomic heterogeneity between different vascular beds suggests that differences in gene expression may determine whether an artery is prone or resistant to atherosclerosis [97].

1.4 Experimental Models of Atherosclerosis

1.4.1 Experimental Model of Atherosclerosis *in Vitro*

Given that VSMCs are critically involved in atherosclerosis development by losing quiescent phenotype and migration into the intimal layer of the vascular wall, these cells represent a suitable *in vitro* model for atherosclerosis [25]. Techniques to explant VSMCs from human or animal vessels are very established and allow studying these primary cells *in vitro* [98-100]. Stimulation of VSMCs with mitogenic stimuli is a common method to simulate an atherogenic environment [25, 98]. The use of VSMCs *in vitro* is an established procedure and may give the possibility to study important molecular aspects of the disease. However, to study cellular complexity and chronic aspects, atherosclerosis development has to be analyzed *in vivo* or *ex vivo* conditions.

1.4.2 Experimental Model of Atherosclerosis *in Vivo*

Knowledge about atherosclerosis development and progression in human *ex vivo*, stems from autopsy studies [101] or, more recently, from intravascular *in vivo* imaging [31]. However, these techniques have limitations as only information about the structure but not about the properties of atherosclerotic lesions is provided [31, 101]. Therefore, the generation of a good animal model of atherosclerosis, which mimics human plaque structure and development, is very important. Such a model allows better understanding the complexity of this chronic disease and, at the same time, provides a tool for the development of therapeutic approaches.

At the beginning of atherosclerosis research, the disease had been studied mainly in primates and rabbits [102]. However, these animals have a long gestation time, low number of offspring and high costs of maintenance, and are therefore not considered as good animal models [103].

An excellent mammalian system to study human diseases afflicting the cardiovascular, the neuronal, the endocrine and the immune system are mice. Mice have the ability to reproduce fast (reproduction time: every nine weeks) and their cost of maintenance is relatively low. However, in atherosclerosis research mice show some limitations: in fact, these animals, in contrast to humans, have high levels of anti-atherosclerotic high density lipoproteins (HDL, > 80 mg/dl) in the blood and low levels of highly atherogenic LDL (< 50 mg/dl) [104]. For these reasons mice are normally resistant to atherosclerosis. Dietary treatment or/and genetic manipulation involved in lipid metabolism are common methods used to attain plaque development in these animals. The commonly used mouse models of atherosclerosis and the characteristic of each model is listed in (**Table 1**) [103, 105].

The most widely used *in vivo* model of atherosclerosis is the apolipoprotein E-deficient ($ApoE^{-/-}$) mouse. These animals spontaneously develop hypercholesterolemia and develop the different lesions-stages observed during atherogenesis in humans [106, 107]. In $ApoE^{-/-}$ mice atherosclerotic plaques increase and advance to more complex fibrous plaques with age. However, these mice do not spontaneously develop plaque rupture [29, 108].

In $ApoE^{-/-}$ mice, as well as in humans, predilection sites throughout the arterial tree exist, which are more susceptible to atherosclerotic lesions formation. The aortic root, the aortic arch, thoracic aorta, the brachiocephalic trunk, the pulmonary artery and regions around the carotid bifurcation are more susceptible to plaque formation [106, 109, 110]. Interestingly, the common carotid artery of these mice was shown to be protected from plaque development [111].

Model	Model description	Diet	Cholesterol level (mg/dl)	Lesion type	Reference
C57BL/6	C57BL/6 is the most widely used mouse strain, due to the availability of congenic strains, easy breeding and robustness	Very high-cholesterol/fat diet containing cholic acid	200 to 300	Fatty streak Plaque in aortic root	103 112
ApoE$^{-/-}$	Apolipoprotein E is a surface constituent of lipoproteins which serves as a ligand for the cell-surface-expressed LDL-receptor, thereby promoting the uptake of LDL particles from the circulation. Deletion of the ApoE gene in mice results in a delayed clearance of lipoproteins resulting in increased LDL plasma levels	Standard diet High-cholesterol/fat diet	400 to 600 1500 to 2000	Fatty streak and fibrous plaques similar to human Same as with standard diet but with faster progression and larger plaques	106, 107,113 113, 114
ApoE 3Leiden	In humans, the rare ApoE 3Leiden allele is associated with dyslipidemia. In ApoE 3Leiden transgenic mice the mutated human apoE3 gene has been introduced	Very high-cholesterol/fat diet containing cholic acid	1600 to 2400	Fatty streak and fibrous plaques	115
ApoE R142C	Mice transgenic for the mutated human ApoE gene (in which the arginine residue at position 142 is mutated to cysteine)	Very high-cholesterol/fat diet containing cholic acid	370	Fatty streak	103
LDL receptor $^{-/-}$	LDL receptor is a transmembrane protein expressed at the cell surface, which bind LDL-associated apolipoproteins to remove LDL from the circulation.	Very high-cholesterol/fat diet containing cholic acid	1500	Fatty streak and necrotic core	116

Table 1: Summary of the commonly used mouse models of atherosclerosis.

1.5 Candidate Gene Affecting Atherosclerosis

To understand the molecular mechanisms which are involved in complex diseases such as atherosclerosis information about changes in gene expression profiles under disease conditions is important.

cDNA microarray technology provide the possibility to identify gene clusters involved in the development of the disease based on transcriptional changes [117]. In **Table 2** different microarray approaches using cells, animal or human samples are listed.

Cell culture studies			
Model	**Intervention**	**Prominent gene pathways**	**Reference**
Murine A404/P19 smooth muscle cells	Differentiation to smooth muscle	Differentiation, development, chromatin remodeling, inhibin βA	118
Human saphenous vein and coronary artery endothelial cells	Comparison of baseline differences, difference with stimulation by oxLDL, cytokines	Regulation of cell growth, oxidoreductase activity and stress, immune and anti-inflammatory responses, fibrinolysis, thrombogenesis	97
Human saphenous vein and coronary artery SMC	Comparison of baseline differences, difference with stimulation by oxLDL, growth factors	α-Chemokines, proinflammatory cytokines, apoptosis, inflammation, lipid biosynthesis, some β-chemokines, metalloproteinase inhibitors	119
Rat aortic SMC	Sodium butyrate (antiproliferative agent)	Cell growth, differentiation, stress response, vascular function	120
Human monocytic THP-1 cells	PMA for differentiation, stimulation with oxLDL, acLDL, LDL	Early response genes (transcription factors), upregulated early; cell proliferation, migration, inflammation, and lipid metabolism activated late	121
Animal models			
Murine aortic tissue	$ApoE^{-/-}$, genetic background, high-fat diet, disease, diet, age	Inflammatory response, TH1, TH2, wound healing, ossification, proteo- and peptidolysis, apoptosis, nitric oxide-mediated signal transduction, cell adhesion and migration	122
Murine aortic tissue	$ApoE^{-/-}$,C57Bl6, genetic background	Growth, differentiation, inflammation, catecholamine synthesis, phosphatase activity, peroxisome function, IGF activity	123

Murine aortic tissue	ApoE$^{-/-}$,C57Bl6	Lipid and lipoprotein metabolism, defense response, inflammation, nuclear organization and biogenesis, morphogenesis	124
Human tissues			
Model	**Intervention**	**Prominent gene pathways**	**Reference**
Human aortas (transplant donors)	Risk factors	Prediction gene set for disease severity (includes ApoE, osteopontin, lor1, also capg, gm2, mmp9, ccrl2)	125
Human arteries	Risk factors	HMG-CoA overexpression in macrophage-rich lesions	126
Human arteries	Risk factors	Known (ApoE, CD68, TIMP, and phospholipase D) and unknown genes (JAK-1, VEGF receptor-2)	127
Human coronary arteries (explanted hearts)	Risk factors	SM dedifferentiation, inflammation	128
Human coronary atherectomy specimens	Risk factors	Inflammation greater in *de novo* lesions	129
Human carotid plaques, transplant donors (media)	Risk factors	Loss of RGS5 from fibrous cap cells	130
Carotid/ mammary arteries	Risk factors	Death-associated protein (DAP) kinase	131

Table 2: Changes in gene expression during atherosclerosis as analyzed by microarray technology. Table was modified from [132].

Changes in expression of genes known to be involved in inflammatory pathways are frequently detected in the microarrays further confirming the importance of inflammation during atherogenesis. Additionally, changes in expression of genes involved in regulation of proliferation, differentiation, lipid-metabolism and apoptosis have been identified. As microarray studies today cover whole transcriptomes, identification of genes so far unrelated to atherogenesis is possible. An example is DAP kinase which was known to be involved in apoptosis but not in atherogenesis [131].

1.5.1 Identification of a Secretase as a Candidate Gene Protecting From Atherosclerosis

Based on his observation made in the mid-1990s that despite severe hypercholesterolemia the common carotid artery of ApoE knockout mice is protected from atherosclerosis compared to the thoracic aorta [111], in 2001 Barton and co-workers performed a microarray study comparing the gene expression profile of the two different vascular beds. In this study Barton e*t al* observed a 3-fold higher expression of β-site APP-cleaving enzyme 2 **(**BACE2) in the atherosclerosis protected carotid artery (Barton M, 2001, unpublished data). This result suggested a possible protective role of BACE2 in atherosclerosis and formed the basis of the present study.

BACE2 is a secretase known to be responsible for ectodomain shedding of the amyloid precursor protein (APP) and other transmembrane proteins [133, 134]. BACE2 is the homologue of β-site APP-cleaving enzyme 1 **(**BACE1). This secretase enzyme is known to cleave APP at the β-secretase recognition site, generating a soluble peptide called amyloid β (Aβ), which accumulates in the brain causing neurodegeneration in Alzheimer diseased patients [135, 136].

Even though Alzheimer's disease and atherosclerosis affect predominantly different organs and tissues, the two diseases share similarities. Common risk factors for vascular diseases and Alzheimer's disease are hypercholesterolemia, hypertension, diabetes, estrogen deficiency and aging [137]. Additionally, inflammation contributes to the pathogenesis of both diseases [19, 138], and drugs used in patients suffering of atherosclerosis, such as lipid lowering agents, may also reduce the incidence of neurodegeneration [139, 140].

Atherosclerosis and neurodegeneration are often occurring combined and narrowing of the cerebral arteries, due to atherosclerosis, is more pronounced in Alzheimer's disease than in nondemented control patients [141].

1.6 Biological Importance of Secretases

Secretases are enzymes which belong to the protease family [142]. Proteases catalyze the cleavage of peptide bonds by hydrolization. Like all enzymes, proteases bind to their substrates only shortly before catalyzing a reaction and releasing the product [143]. Proteases can be divided in endopeptidases, which cleave internal peptide bonds, and exopeptidases, which cleave the terminal peptide bonds. Exopeptidases cleave at the amino (aminopeptidases) or at the carboxy (carboxypeptidases) terminus [144]. By means of their catalytic residues which are essential for their enzymatic activity, proteases can also be

classified in 6 groups: Serine proteases, which contain a serine together with a aspartic acid and a histidine residue in the active site of the protein (e.g. trypsin, elastase, thrombin), threonine proteases, with a threonine residue in the catalytic site (e.g. the proteasome catalytic subunits), cysteine proteases, which have a cysteine, a histidine and an aspartic acid as their catalytic site (e.g. papain), aspartyl proteaseas, which are enzymes with two aspartic acid residues in the catalytic site (e.g. BACE1, BACE2 and pepsin), glutamic acid proteases, which have glutamic acid as catalytic active-sites and finally metalloproteases, which have glutamic acid, tyrosine and a metal ion (usually zinc) in the catalytic active site [e.g. matrix metalloproteases and proteins of the A disintegrin and metalloproteases (ADAM) family] [144].

Membrane protein proteases, which have the capability of cleaving (shedding) integral membrane proteins generating soluble forms, are called secretases (or sheddases). Secretases cleave their substrate close to the membrane surface at defined positions of the protein, which is determined by secretase specificity and by the topology of the substrate. The determinants and factors that result in a transmembrane protein becoming a substrate of secretases is still unclear [142]. Secretases can only proteolytically cleave type I and type II transmembrane proteins which have an accessible cleavage region close to the membrane surface [145].

The biological function of secretases is highly variable. Depending on the biological context and on the transmembrane protein cleaved, proteolysis may have either positive or negative physiological outcome. Since secretases are highly specific modulators of different signaling pathways involved in many pathophysiological processes such as neurodegeneration, apoptosis, oncogenesis and inflammation, these enzymes are considered of high biological and therapeutical importance [146]. Depending on the substrate cleaved, different biological functions are mediated (**Figure 8**):

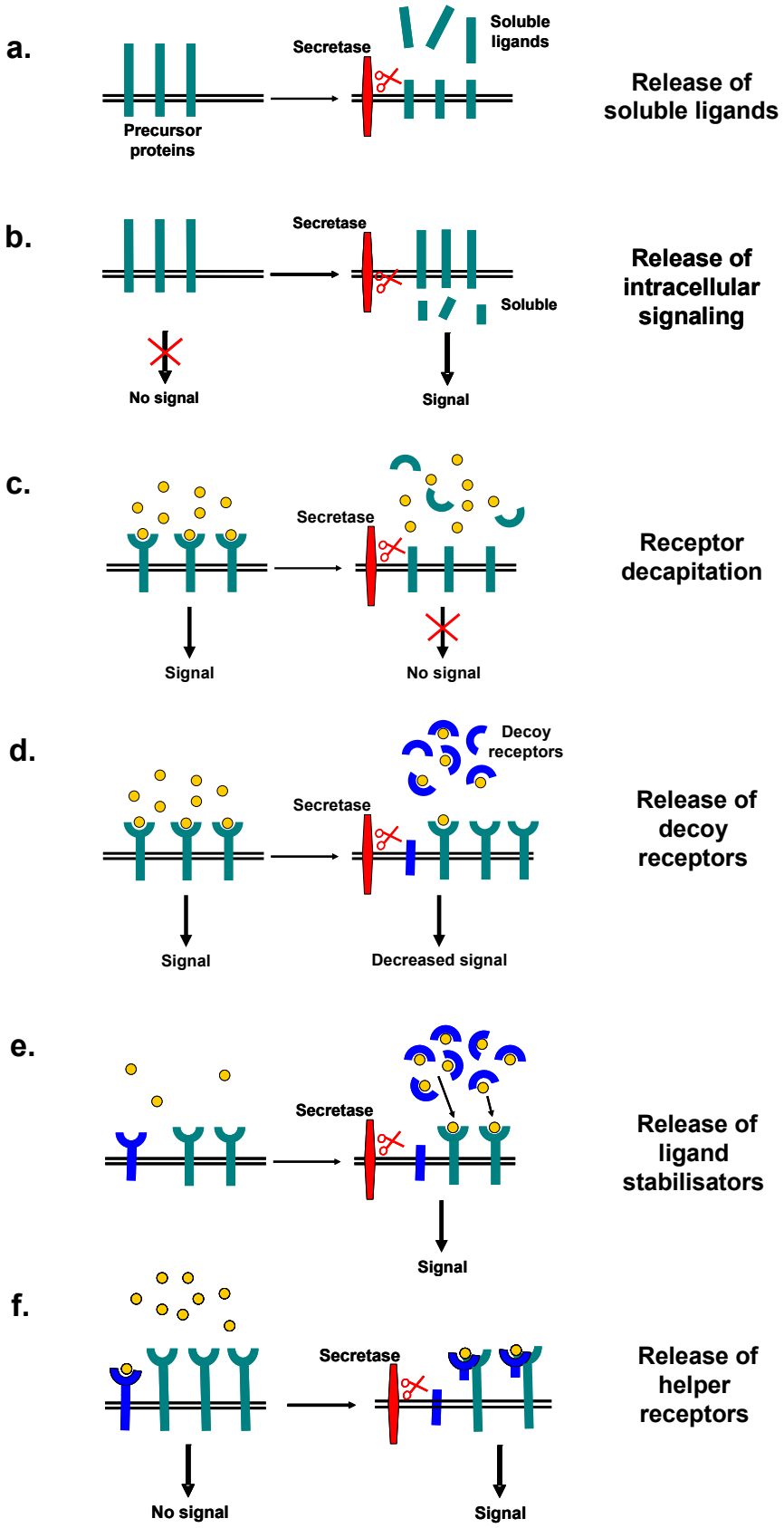


Figure 8: Biological function of secretases. Representation of different biological outcomes induced by secretase-mediated proteolytic cleavage of transmembrane proteins (**a-b**) and transmembrane receptors (**c-f**).

a. Release of Cytokines and Growth Factors in the Extracellular Space or in the Blood

TNF-α [147] and transforming growth factor α (TGF-α) [148] are examples for proteins synthesized as transmembrane precursor, which are released by proteolysis from the cell into the blood or into the extracellular space [149] (**Figure 8a**). Membrane-anchored precursors may be biologically active, having the capability of binding to an adjacent receptor in that form [148]. Additionally, the membrane anchored precursor and the soluble ligand may show different receptor specificity and therefore different function [150].

b. Initiation of Intracellular Signaling Pathways via Regulated Intramembrane Proteolysis of Transmembrane Proteins

In this case the shedded peptide is released into the intracellular space and moves into the nucleus to regulate expression of different genes (**Figure 8b**). This mechanism is for instance known during Notch signaling [151].

c. Down-Regulation of Membrane Protein From the Cell Surface

Proteolysis of transmembrane proteins may serve as post-tranlational modification to rapidly downregulate the protein from the cell surface. If the transmembrane protein cleaved by the secretase is a transmembrane receptor, cleavage may serve to prevent the signaling cascade into the cell (**Figure 8c**). This process, also called "receptor decapitation", was observed for transferrin receptor [152].

d. Release of Decoy Receptors

Cleavage of transmembrane receptors may also lead to the secretion of soluble forms. Frequently, soluble receptors compete with their membrane-bound counterpart for their ligand, and thereby acting as competitive antagonists or "decoy receptors" [146] (**Figure 8d**). The extracellular domain of IL-1 receptor type II (IL-1RII) is for example released by metalloproteases. The soluble form competitively binds IL-1, thereby reducing the amount of pro-inflammatory cytokines able to bind and activate IL-1 receptor type I (IL-1RI) and therefore resulting in inflammation [153].

e. Release of Ligand Stabilizers

Soluble receptors secreted by proteolysis may also have agonistic function by protecting a ligand from degradation/clearance (**Figure 8e**). Growth hormone binding protein is known to

be shedded and to complex as soluble form with growth hormone, resulting in 10-fold lower clearance of the ligand [154].

e. Release of Helper Receptors

A secreted receptor may increase or facilitate the ligand signaling capability into the target cell (**Figure 8f**). The cytokine IL-6 forms a complex with its soluble receptor allowing binding to a further signal transducing receptor [155].

As secretases are involved in so many biological contexts, expression, regulation and activity of these enzymes need to be tightly regulated and controlled. Regulation of their transcription and translation is the first step in controlling secretase expression. Furthermore secretases are frequently synthesized as proenzymes (zymogens), which are also preventing an irregular activation of these enzymes. Additionally, endogenous inhibitors are important regulatory mediators [144].

1.6.1 The Secretase Family

The secretase family consists of α- β- and γ-secretases. These three different types of secretases mediate ectodomain shedding of transmembrane proteins at different cleavage sites. α- and β-cleavage results in ectodomain shedding and occurs very close to the transmembrane domain, whereby α-secretases cleave proteins closer to the plasma membrane compared with β-secretases [156, 157]. γ-Secretase cleavage results in intramembrane proteolysis occurring inside the hydrophobic region of the plasma membrane [158]. A number of proteins, including TNF-α [147], TGF-α [148], growth hormone binding protein [154] and Notch [151], undergo α- β- or γ-secretase-mediated cleavage. However, the most studied transmembrane protein shedded by these secretases is APP [157, 159], the key protein involved in senile plaque development in the brain of patient suffering from Alzheimer's disease [135, 136]. APP is a 770 amino acid containing type I transmembrane protein, which is ubiquitinously expressed throughout the human body [160]. APP is first cleaved by α- or β- secretases resulting in ectodomain release (**Figure 9**). The remaining membrane-bound region undergoes a second γ-secretase-mediated cleavage resulting in the release of a small extracellular peptide and an intracellular C terminal fragment (CTF). Proteolytic cleavage of APP by β-secretase followed by γ-secretase cleavage, results in the generation of a neurotoxic peptide called amyoid-β

(Aβ) [159]. On the contrary, shedding of APP by α-secretase followed by γ-secretase, produces a smaller peptide (p3), which is not involved in Alzheimer's disease [157] (**Figure 8**).

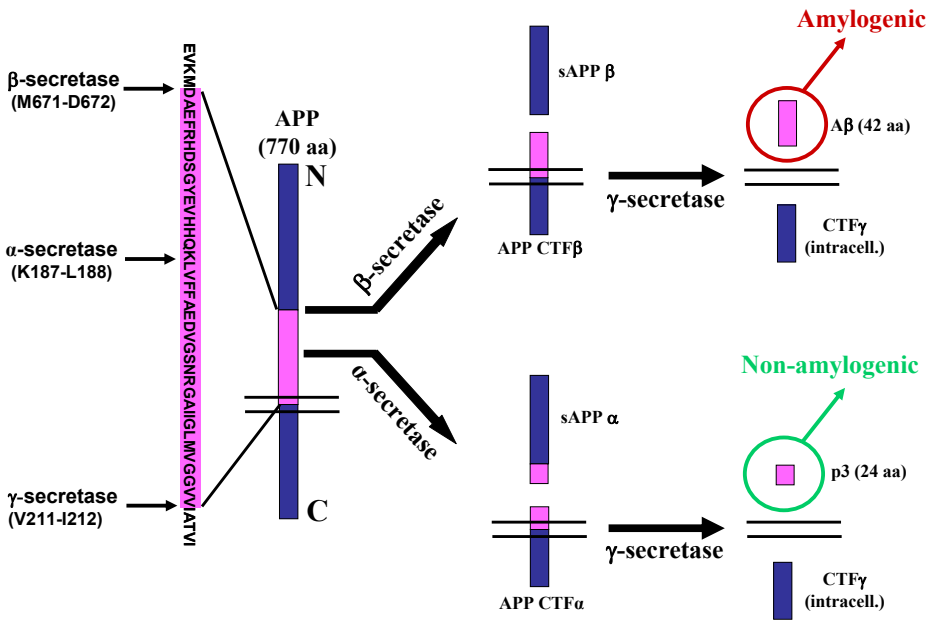


Figure 9: Cleavage sites of α- β- and γ-secretases in the β-amyloid precursor protein (APP). The 770 amino acid (aa) transmembrane protein APP is schematically represented. The amino acid sequence of the region cleaved by the secretases is amplified. The region which represents the highly amylogenic peptide Aβ is colored in pink.
Cleavage of APP by β-secretase followed by γ-secretase results in the release of a 42 amino acids Aβ, which is known to accumulate in the brain causing neurodegeneration. α-Secretase cleavage prevents the generation of the amylogenic peptide by cleaving APP in the middle of the Aβ domain.

α-Secretase: The transmembrane proteins belonging to the ADAM family are α-secretases [161]. Proteins belonging to this family like TNF-α-converting enzyme (also called ADAM-17) [162], ADAM-10 [163], ADAM-9 [164] and ADAM-19 [165] are able to cleave APP. BACE2 is considered to be an alternative α-secretase [166, 167].

α-Secretases cleave APP predominantly in the middle of the Aβ domain between lysine 187 and leucine 188 (K187-L188) [157] , resulting in the generation of a soluble protein (sAPP α) and a shorter membrane-bound 83 residues APP C-terminal fragment (APP CTF α) [157]. The subsequent γ-secretase-mediated cleavage of APP CTF α releases CTFγ in the intracellular space and a much shorter (24 amino acids) extracellular peptide called p3, which is non-amylogenic [157] (**Figure 9**).

β-Secretase: The β-secretase cleavage enzyme 1 (BACE1) has been identified as the major β-secretase responsible of APP cleavage *in vivo* [136]. BACE1 is a type I transmembrane aspartyl protease with two important catalytic active aspartic residues at the N-terminal end of the protein. BACE1 is highly expressed in the brain, especially in neurons, and proteolytic cleavage of APP by BACE1 followed by γ-secretase cleavage is resulting in the generation of neurotoxic Aβ peptide [159]. BACE1 cleaves APP predominantly between methionine 671 and aspartic acid 672 (M671-D672), generating a soluble protein (sAPP β) and a 99 residues long membrane-bound APP CTFβ. APP CTFβ undergoes a subsequent intramembraneous cleavage catalyzed by the γ-secretase enzyme, resulting in release of an intracellular fragment (CTFγ) and an extracellular 42 amino acid peptide, Aβ, which accumulates in the brain leading to neurodegeneration [136] (**Figure 9**). A different isoform of this secretase, BACE2, has also β-secretase cleavage capability. However BACE2 cleaves at this position less effectively than BACE1 [134].

γ- Secretase: Cleavage of the C-terminal fragment which still remains bound to the membrane after α- or β-secretase cleavage is the last step for the generation of the non-amylogenic p3 or the highly amylogenic Aβ peptide, respectively. This final cleavage is accomplished by γ-secretases. These special enzymes are integral membrane proteins with the ability to cleave single-pass transmembrane proteins inside the hydrophobic environment of the cell membrane. After γ-secretase cleavage of the CTF of APP, the generated small peptides p3 or Aβ are secreted from the cell, whereas a CTF γ is released in the intracellular space [157] (**Figure 9**). If the CTF γ has some transcriptional regulatory role, as shown for Notch intracellular domain [151], is still unclear. Two homologous proteins, presenilin 1 and 2, are responsible for the γ-secretase cleavage of APP [168].

1.6.2 β-site APP-Cleaving Enzyme 2

β-site APP-cleaving enzyme 2 (BACE2, also called Asp1) was cloned in 1999 [134, 136, 169, 170] and was mapped on human chromosome 21q22.3, the Down's syndrome critical region. The gene codes for a 518 amino acid containing type I transmembrane protein with aspartyl protease activity. BACE2 is composed of 9 exons, two putative *N*-glycosylation sites and two catalytic aspartic acid residues at exon 2 and 6 respectively [134].

The N-terminal part of the protein is composed of a small 22 amino acid signal peptide, which is important for its trans-membrane localization during protein translation in the

endoplasmatic reticulum. The signal peptide is followed by a 40 amino acid pro-peptide region, which is proteolitically cleaved in order to generate a mature BACE2 protein [171] (**Figure 10**). The pro-peptide of BACE2 was demonstrated to be removed autocatalytically [166, 172]. In neuronal and fibroblast cells BACE2 localizes in the plasma membrane and throughout all secretory membrane compartments, including the Golgi apparatus/trans-Golgi network (TGN) and early endosomes [166, 171, 172]. *N*-glycosylation and protease activity of BACE2 was shown to occur in the acid environment of the Golgi compartment [171, 173].

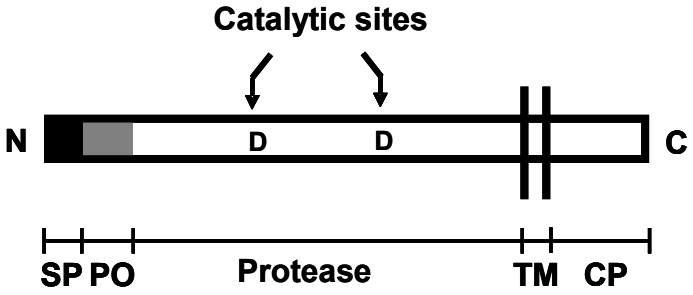


Figure 10: Schematic representation of BACE2 domain structure. BACE2 is represented from the N- to the C- terminus. Signal peptide- (SP), Pro- (PO), Protease-, Transmembrane- (TM) and Cytoplasmic- (CP) domain are represented. The two essential aspartic (D) catalytic residues for the secretase activity are indicated.

BACE2 shares 51% homology at the amino acid level with BACE1, showing a particular high homology in the catalytic region [134]. However, the regulatory domains of the two promoter regions do not show high similarity [163], and the promoter activity of BACE2 shows reduced function in neuronal cells compared with BACE1 promoter [174]. Rather, BACE2 is expressed at low level in the brain but is found to be highly expressed in vascularized tissues like liver, kidney, pancreas, placenta and heart [134]. A role of BACE2 in the pathogenesis of inflammatory muscle disease in humans has also been suggested [175].

BACE2, like its homologue, mediates ectodomain shedding of APP [134]. However, this secretase cleaves the protein at a different preferential site [166, 167]. Whereas BACE1 is the main enzyme cleaving APP at the β-secretase site, BACE2 has limited cleavage capability at that position but exhibits mainly α-secretase-like activity. BACE2 proteolytically cleaves in the middle of the Aβ peptide sequence between phenylalanine 190 and phenylalanine 191 (F190-F191) efficiently, which is 3 amino acids after the α-secretase cleavage site. For this reason BACE2 is also called alternative α-secretase [166], and cleavage of APP by this protein followed by γ-secretase proteolysis results, like for the α-secretase cleavage, in a small p3

peptide, which precludes the formation and accumulation of Aβ in the brain [167]. Furthermore, BACE2-transfected cells produce reduced level of Aβ [167, 176, 177] and knock-out of the gene by siRNA resulted in elevated secretion of the amylogenic peptide [167, 176]. These results strongly suggest the limited cleavage capability of BACE2 as a β-secretase and its function in precluding the generation of the amylogenic peptide. Thus, BACE1 and BACE2, even being highly homologue, have antagonistic effects in the development of Alzheimer's disease [166, 167, 177]. In order to better understand the biological function of BACE2 *in vivo*, BACE2 knock-out mice were also generated. Interestingly, these mice are fertile, viable and do not show major phenotypic abnormality [176].

Besides APP, IL-1R2, CD16 and TGF-α are also substrates of BACE2 [133]. Cleavage of IL-1R2 occurs in a manner similar to APP, resulting in the secretion of IL-1R2 ectodomain and the generation of a CTF, which undergoes further γ-secretase proteolysis [133]. Functions on cell growth or inflammatory pathway mediated by BACE2 have not been described up to now.

1.7 Aim of the Project

Based on the identification of BACE2 by microarray in the vascular wall of an atherosclerosis-protected artery, this study investigated the potential role BACE2 as a novel modulator of human vascular smooth muscle cell (VSMC) function *in vitro* and atherosclerosis *in vivo*.

Specifically, the aim of the present study was:

1. to characterize the expression, intracellular localization, and expressional regulation in human VSMCs.
2. to determine potential mechanisms by which BACE2 interferes with human VSMC function and growth under stimulated conditions.
3. to determine the effects of BACE2 overexpression on atherogenesis *in vivo*.

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2. MANUSCRIPT DRAFT

Characterization of BACE2 as a Modulator of Vascular Smooth Muscle Cell Function and Atherosclerosis

ABSTRACT

Atherosclerosis is a chronic inflammatory disease, which is associated with proliferation of vascular smooth muscle cells (VSMCs). β-site APP-cleaving enzyme 2 (BACE2) is an aspartyl protease with unknown function. In this study we characterize the expression, localization, regulation and function of BACE2 in human VSMCs in vitro and the effect of BACE2 on experimental atherosclerosis in vivo. We demonstrate that BACE2 is localized in caveolae, membranes of secretory pathway and plasma membrane of VSMCs. Growth factor stimulation results in a rapid proteasome-dependent degradation of BACE2. Overexpression of BACE2 inhibits cell proliferation, and is accompanied with downregulation of c-jun- and IκBα-dependent pro-inflammatory signaling. Furthermore, we generated transgenic $ApoE^{-/-}$ mice overexpressing BACE2. In these mice the amount of atherosclerotic plaques and blood plasma concentrations of the pro-inflammatory cytokine IL-1β, cholesterol and lipids are reduced. These results suggest a protective role of BACE2 in atherosclerosis.

INTRODUCTION

Atherosclerosis is a chronic inflammatory vascular disease and the most common cause of death in Western countries [1]. High plasma level of low density lipoproteins (LDL) is an important risk factor contributing to the initiation and progression of the disease [2]. Atherosclerotic plaques are composed of smooth muscle cells (VSMCs), macrophages, lipids, T-cells and fibrous material [3]. In the development of atherosclerotic plaques, VSMCs have been shown to play a central role, particularly in the initiation of the disease [4]. In healthy vessels, VSMCs are located in the medial layer of the arterial wall and are fully differentiated, showing a quiescent contractile phenotype. In diseased condition, however, these cells undergo phenotype switching resulting in dedifferentiation and migration from the medial into the intimal layer where the cells proliferate, secrete high amounts of cytokines, accumulate and start to synthesize extracellular matrix, resulting in thickening of the arterial intimal layer and formation of fibrous caps [5]. The underlying mechanisms of phenotype switching in VSMCs are complex [6]. Proliferation of VSMCs in the arterial wall is known to be stimulated by growth factors, cytokines and bioactive peptides [7]. Generally, the stimulatory molecules bind to cognate receptors from which a variety of kinase-dependent signal cascades are initiated: insulin receptor substrate 1 (IRS-1)- phosphoinositide-3 kinase (PI3K)- protein kinase B (Akt)- glycogen synthase kinase 3 α/β (GSK3) [8], cAMP response element-binding protein (CREB) [9], 70 kDa ribosomal S6 kinase (p70S6 kinase) [10], canonical IκB kinase (IκB) [11] and mitogen-activated protein kinases (MAPK) [12]. Among others, dimeric transcription factors such as activator protein-1 (AP-1) [13], of which c-Jun is a component, and nuclear factor-κB (NF-κB) [11] are targets of these cascades. These transcription factors have been shown to be integrators of inflammatory processes [6, 14, 15], which are associated with phenotype switching and subsequent proliferation of arterial VSMCs [6, 7, 16].

The aspartyl protease BACE2 (β-site APP-cleaving enzyme 2) is an alternative α secretase which mediates ectodomain shedding of amyloid-β precursor protein (APP) and of other transmembrane proteins [17, 18]. Its homologue β-site APP-cleaving enzyme 1 (BACE1), a β-secretase which is predominantly expressed in neuronal tissues [19, 20], has been demonstrated to be the rate limiting enzyme in the formation of the neurotoxic amyloid-β (Aβ) from APP. This process is the key event in senile plaque development in brains of patients suffering from Alzheimer's disease [19, 20]. Proteolytic cleavage of APP by BACE1 at the β-secretase recognition site results in generation of a soluble APP (sAPPβ) and a transmembrane C-terminal fragment-β (CTFβ). The neurotoxic Aβ fragment is generated during a subsequent

intramembrane proteolytic step catalyzed by a γ-secretase protease complex. BACE2, however, preferentially cleaves APP at alternative α-secretase recognition sites [21-23] leading to the formation of a smaller APP product called p3, which precludes the formation and accumulation of Aβ [21, 22]. Moreover, BACE2 is expressed at low level in the brain but is found to be highly expressed in vascularized tissues as shown by northern blot analysis [17]. In previous work in this laboratory Barton and coworkers identified BACE2 to be highly expressed in arteries using cDNA microarray technology (M. Barton, unpublished observation 2001 and manuscript in preparation). Thus, we speculated that BACE2 may have a role in the regulation of vascular diseases.

To assess a potential role of BACE2 in atherosclerosis we investigated the expression, localization, regulation and function of BACE2 *in vitro* in VSMCs and *in vivo* using a well established mouse model of atherosclerosis: the apolipoprotein E-deficient mice ($ApoE^{-/-}$). These hypercholesterolemic animals develop all the different lesions-stages observed during atherogenesis in human [14, 24, 25].

In the present study we show that proliferative stimuli negatively regulate BACE2 gene and protein expression in VSMCs. Overexpression of BACE2 resulted in reduced activation and expression of pro-inflammatory signal cascades and cell proliferation whereas BACE2 gene silencing enhanced cell growth. In addition, we generated $ApoE^{-/-}$ BACE2 transgenic mice which, in support to our *in vitro* data, show reduced amount of aortic atherosclerotic plaques, reduced plasma concentration of IL-1β, cholesterol and lipids, suggesting a protective role of BACE2 in atherosclerosis.

MATERIAL AND METHODS

MATERIAL AND METHODS

Materials:

If not otherwise stated all chemicals were obtained from Sigma (Aldrich, Deisenhofen, Germany). Primers and oligonucleotides were synthesized from Microsynth (Balgach, Switzerland).

Site Directed Mutagenesis of BACE2

A mutant (MUT) BACE2 lacking protease activity was generated by mutating the two catalytic aspartic acid residues (D) [23] to alanine (A). The mutations were inserted by site-directed mutagenesis using pCDNA3.1-wild-type-BACE2 as a template and primers containing the mutated sequence (for mutation D110A: forD110A and revD110A primers; for mutation D303A: forD303A and revD303A primers, **Supplementary Table 1**) using a Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instruction.

BACE2 Plasmid Construction

Human wild-type (WT) and mutant (MUT) BACE2 were amplified by PCR using a forward primer containing a *XbaI* restriction site (forXbaI primer, **Supplementary Table 1**) and a reverse primer containing a *BamHI* restriction site (revBamHI primer, **Supplementary Table 1**), using Proof Star DNA Polymerase (Qiagen, Basel, Switzerland) (Q-Solution, 1x Proof Star Buffer, 2.5 mmol/l $MgSO_4$ and 10 mmol/l dNTP). After digestion with *XbaI* and *BamHI*, PCR products were ligated into c-FUW [26], a replication-defective lentivirus vector, using T4 Ligase (Roche, Rotkreuz, Switzerland).

The mammalian expression vector pCAG [27] (**Supplementary Fig. 1a**) was digested with *EcoRI* and treated with alkaline phosphatase, CIAP (Roche, Rotkreuz, Switzerland) to avoid religation. An *EcoRI-SalI-HindIII-EcoRI*-linker oligonucleotide (forLinker and revLinker, **Supplementary Table 1)** was ligated with the linearized expression vector. BACE2 was amplified by PCR using a *SalI*-containing forward primer (forSalI primer, **Supplementary Table 1)** and a *HindIII* containing reverse primer (revHindIII primer, **Supplementary Table 1**) using Proof Star DNA Polymerase (Q-Solution, 10x Proof Star Buffer, 2.5 mmol/l $MgSO_4$

and 10 mmol/l dNTP). Amplicons and pCAG were cleaved with *SalI* and *HindIII* and ligated with T4 DNA Ligase.

All constructs amplified by PCR were verified by sequencing (Microsynth, Balgach, Switzerland).

Cell Culture Procedures

Human embryonic kidney 293T cells (HEK293T) were maintained in Dulbecco Medium (Bioconcept, Allschwil, Switzerland), fetal human aortic smooth muscle cells (FLTRs) in DMEM High Glucose (Biochrom AG, Basel, Switzerland) and human vascular smooth muscle cells (VSMCs) in DMEM Ham's F12 medium (Bioconcept, Allschwil, Switzerland) supplemented with 10% fetal calf serum (FCS, Sigma Aldrich, Deisenhofen, Germany), 2 mmol/l glutamine (Biochrom AG, Basel, Switzerland) and 100 µg/ml penicillin/streptomycin (Gibco, Basel, Switzerland).

VSMCs were explanted from human umbilical veins (an arterial vessel) using the explant technique as described [28, 29]. Human umbilical cords were obtained at the Klinik für Geburtshilfe at the Universitätsspital Zürich. The studies were conforming to the principles outlined in the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Zurich. Informed consent was obtained from all patients. Explanted cells were then cultured in petri dishes and identified by their hill and valley morphology using phase-contrast microscopy and immunofluorescence stained for α-actin [29]. For experiments subconfluent cells at passage 3-6 were used.

Cells were kept at 37°C in a 5% CO_2 incubator and passaged with 0.05% trypsin (w/v)/0.02% EDTA (w/v) in phosphate-buffered saline (PBS, Gibco, Basel, Switzerland).

Before experiments cells were starved with 0.1% FCS for 24 h. Stimulation experiments with 10% fetal calf serum, 20 ng/ml human recombinant platelet-derived growth factor BB homodimer (PDGF; Calbiochem, Dietikon, Switzerland) or 50 µg/ml low-density lipoprotein (LDL, isolated from human EDTA plasma obtained from healthy blood donors as described in [29]) were performed when cells were 70% confluent.

Proteasome inhibitors MG-132 (10 µmol/l, Calbiochem, Dietikon, Switzerland) and Lactacystin (20 µmol/l, Alexis Biochemicals, Lausen, Switzerland) were preincubated for 6 h before stimulation with FCS. 10 and 100 nM of β-secretase inhibitor ($C_{73}H_{118}N_{16}O_{27}$, MP Biomedicals, Illkirch, France) and γ-secretase inhibitor IX ($C_{23}H_{26}F_2N_2O_4$, Calbiochem, Dietikon, Switzerland) were preincubated for 30 min before FCS treatment. All stimulations were terminated by washing cells with ice-cold PBS for further procedures.

Cell Proliferation Assay

Primary VSMCs (50% confluent) were starved for 24 h (0.1% FCS), stimulated by FCS and after 18 h (methyl-^{3}H)-thymidine (3 μCi/ml, 1.5 μmol/l) was added (GE-Healthcare, Zurich, Switzerland). Thymidine incorporation was measured 6 h later using a β-counter as described [30].

Alternatively, a cell counting method was used. Equal number of cells were plated. The following day cells were starved for 3 days followed by 2 days of FCS stimulation. Cells were counted using a hemocytometer immediately before starvation, after 3 days of starvation and after 2 days of FCS stimulation.

Generation of Smooth Muscle Cells Stably Overexpressing Wild-Type and Mutant BACE2

Stable WT and MUT BACE2 overexpressing VSMCs and FLTRs and CTL cells were generated by lentiviral transduction [26]. Duplication-deficient lentiviruses were assembled in HEK293T cells by transiently tranfecting the cells with the generated c-FUW lentivirus constructs [c-FUW(WT BACE2); c-FUW(MUT BACE2) or c-FUW] described above along with two virus packaging plasmids (pHCMV-G and pCMV) using Lipofectamine 2000 (Invitrogen, Basel, Switzerland) in OptiMEM I Reduced Serum Medium (Gibco, Basel, Switzerland) according to the manufacturer's instruction. Cell culture supernatants containing the replication-defective lentiviruses were collected 48 h after transfection, centrifuged for 5 min at 150 g (4°C), filtered (0.45 μm), pipetted onto 70% confluent VSMCs (or FLTR cells) and incubated overnight. Next day supernatants were replaced with fresh cell culture medium. The virus-transduced cells were expanded and tested for overexpression (**Supplementary Fig.2**).

***In Vitro* Assay of BACE2 Proteolytic Activity**

The second day after transfection, cells were washed with PBS and lysed in 10 mmol/l Tris HCl, pH 7.0 as described [31]. Lysates were centrifuged at 15,700 g (4°C) for 15 min and the pellet containing membrane bound proteins was resuspended in 25 mmol/l Tris, pH 4.5. After passing the membrane proteins for 10 times through a 29 g syringe needle, proteins were homogenized for 30 min at 4°C on a rotor. Cell debris was removed by centrifugation at 400 g for 10 min and protein concentration was determined in the supernatant using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Reinach, Switzerland).

An APP-based substrate (Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp, customer-designed by Bachem AG, Bubendorf, Switzerland) corresponding to the peptide sequence of the alternative α-secretase cleavage site targeted by BACE2 [21-23], and containing a fluorescence donor and a quencher acceptor at each end was used. The full-length substrate is only weakly fluorescent but becomes highly fluorescent after proteolytic cleavage (fluorescence resonance energy transfer (FRET)-principle). Activity of the lysates (5 µg) was measured in an *in vitro* assay after addition of 10 µg of the substrate at 37°C and by reading fluorescence at 320 and 420 nm excitation and emission wavelengths, respectively using Spectra Max M2 microplate reader (Bucher Biotec, Lucerne, Switzerland) machine. Background fluorescence was subtracted.

RNA Extraction and Quantitative Real-Time PCR

RNA was isolated following the manufacturer's instruction (Qiagen, Hilden, Germany). Total RNA was extracted using either the RNeasy Micro kit (VSMCs), the RNeasy Micro kit for RNA isolation from fibrous tissues (aorta, carotid, heart and muscle), or the RNeasy Mini kit for RNA isolation from animal tissues (liver, kidney, brain, lung and spleen).

Purity of RNA was controlled by RT(−) reactions (PCR with non-transcribed RNA). RNA from the different murine tissues was reverse-transcribed with the QuantiTec Reverse Transcription kit (Qiagen, Hilden, Germany), whereas RNA extracted from human primary cells was reverse-transcribed with the Omniscript RT kit (Qiagen, Hilden, Germany).

Real-time quantitative PCR was used to determine steady-state mRNA expression using human (**Supplementary Table 2)** or mouse **(Supplementary Table 3)** primers**.** Two-step PCR was performed with iQ SYBR Supermix PCR kit (Bio-Rad Laboratories, Reinach, Switzerland) as follows: activation of the hot start *Taq* polymerase for 3 min (95°C), followed by 40 cycles of denaturation at 95°C for 15 sec (step 1), and annealing and extension at 60°C for 1 min (step 2). Fluorescence was detected at the end of each extension step. Identity and specificity of amplicons were confirmed by agarose gel electrophoresis, melting curve analysis and sequencing. Gene expression was calculated using the $2^{-\Delta CT}$ method [32]. The relative amount of each mRNA was normalized to the housekeeping gene β-actin.

Protein Extraction and Western Blot

Cells were placed on ice, washed with ice-cold PBS and proteins were extracted with lysis buffer (1% NP-40, 100 mmol/l NaCl, 25 mmol/l β-glycerophosphate, 20 mmol/l Tris HCl pH 7.5, 1 mmol/l EDTA, 1 mmol/l Na-PP_i, 1 mmol/l $NaVO_3$) at 4°C. Extracts were homogenized

for 30 min on a rotor at 4°C, followed by centrifugation at 15,700 g (4°C) for 10 min. Supernatants were collected and protein concentrations were measured. Equal amounts of proteins (20-30 µg total protein) were separated by electrophoresis with 4-12% SDS/PAGE gradient gels (Invitrogen, Basel, Switzerland) and blotted onto a nitrocellulose membrane (GE-Healthcare, Zurich, Switzerland). The membranes were incubated for 1 h in blocking solution (20 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 0.1% v/v Tween-20, and 1% w/v non-fat milk powder) and overnight at 4°C with primary antibodies, diluted 1:1000 or 1:500 in blocking solution. After washing the membrane with blocking solution, the appropriate horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse (GE-Healthcare, Zurich, Switzerland) or anti-goat (Santa Cruz Biotechnology, LabForce, Nunningen, Switzerland) IgG secondary antibodies were added at dilutions 1:5,000; 1:2,000 or 1:10,000, respectively, to the blocking solution for 45 min at room temperature. After washing the membrane with blocking solution and Tris buffered saline (TBS), the immunoreactive bands were visualized using enhanced chemiluminescence (ECL, GE-Healthcare, Zurich, Switzerland). The following primary antibodies were used: goat anti-BACE2, mouse anti-α-tubulin, rabbit anti-$G_{\alpha q/11}$ (Santa Cruz Biotechnology, LabForce, Nunningen, Switzerland), mouse anti-caveolin 1 (BD Biosciences, Basel, Switzerland), rabbit anti-phospho-(Ser473)-Akt, rabbit anti-phospho-(Thr202/Tyr204)-ERK1/2 MAPK (Cell Signaling, Boston, USA).

RNA Interference of BACE2

siRNA Oligonucleotides used in this study are: sense, ACAGAGAGGUCUAGCACAUtt; antisense, AUGUGCUAGACCUCUCUGUtt. These oligonucleotides specifically mediated degradation of BACE2, but not of BACE1, mRNA [21]. Annealed siRNA (25 pmol/ml) was diluted in Opti-MEM I Reduced Serum Medium and transfected into VSMCs at 40-50% confluency overnight using Lipofectamine 2000 as described in the manufacturer's instructions. As negative control a scramble siRNA (siCONTROL, Dharmacon, Lausanne, Switzerland) was used. After transfection the cells were incubated at 37°C (5% CO_2) for 24-94 h prior to testing gene silencing.

Fractionation of Cytosolic and Membrane Proteins

Cell fractionation was performed as described [33] with minor modifications. The pelleted cells were resuspended in buffer containing protease inhibitors (25 mmol/l HEPES, 1 mmol/l EDTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 100 µmol/l phenylmethylsulfonylfluoride) and passed for homogenization 10 times through a 29g syringe needle followed by 10 min

centrifugation at 100 g (4°C). The supernatant containing total cell lysates was ultracentrifuged for 1 h at 55,000 g (4°C). The resulting supernatant was referred to as cytoplasmic fraction. For solubilization of membrane proteins the pellet was further resuspended in triton X-100 containing buffer (20 mmol/l HEPES, 2 mmol/l EDTA, 50 mmol/l β-glycerolphosphat, 1 mmol/l dithiothreitol DTT, 1 mmol/l Na_3VO_3, 1% Triton-X100, 10% glycerol, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 100 µmol/l phenylmethylsulfonylfluoride) and homogenized as before followed by 1 h ultracentrifugation at 55,000 g (4°C). The supernatant was referred to as triton-soluble and the pellet resuspended in loading buffer as triton-insoluble membrane fraction.

Immunofluorescence Analysis

Cells were cultured on sterile coverslips till 70% of confluency was reached. Cells were washed with cold PBS and fixed for 10 min in 4% Paraformaldehyde (PFA, Sigma Aldrich, Deisenhofen, Germany) at room temperature. Cells were permeabilized for 5 min with 0.05% Triton X-100 in PBS (Sigma Aldrich, Deisenhofen, Germany) and washed three times (for 10 min), respectively, with PBS, 50 mmol/l NH_4Cl (in PBS) pH 7.2 and again with PBS. After blocking for 30 min with 5% donkey serum (DS, Jackson ImmunoResearch, West Baltimore, USA) coverslips were transferred to a silicon chamber and incubated overnight at 4°C with primary antibodies against BACE2 and against cell compartment specific proteins. The following day cells were washed with 5% DS followed by TRITC- or Cy5-labelled secondary antibody/ies incubation for 1 h at room temperature. All antibodies were diluted 1:100 in 5% DS. After washing the cells, slips were mounted onto a microscope slide using Moviol-solution (Calbiochem, Dietikon, Switzerland). Fluorescence-labeled proteins were visualized by confocal microscopy (Leica SP2) at 550 nm-570 nm excitation and emission wavelengths, respectively for TRITC and at 650 nm-670 nm for Cy5. Images were processed using Adobe® Photoshop®. The following antibodies were used: rabbit anti-BACE2 (Zymed Laboratories, San Francisco, USA), mouse anti-Early endosomal antigen 1 (EEA1), mouse anti-CD107a, mouse anti-caveolin 1, mouse anti-syntaxin (BD Biosciences, Basel, Switzerland), anti-rabbit TRITC and anti-mouse-Cy5 (Jackson ImmunoResearch, West Baltimore, USA).

Flow Cytometry Analysis

Cells were fixed with 1% PFA for 20 min at room temperature. Cells were permeabilized by incubation with 0.05% Triton X-100 in PBS for 10 min to determine total BACE2, or left unpermeabilized to identify BACE2 localized on the plasma membrane. After washing with

PBS, cells were incubated with an antibody recognizing the N-terminus of BACE2 protein (rabbit anti-BACE2; Serotec, Oxford, UK) for 1 h at room temperature. After washing, cells were incubated with anti-rabbit FITC-conjugated secondary antibody (Jackson ImmunoResearch, West Baltimore, USA) for 30 min at room temperature, fixed again with 1% PFA for 10 min and analyzed by flow cytometry at 492 nm excitation and 520 nm emission (FACSCalibur, BD Biosciences, Basel, Switzerland). Antibodies were diluted 1:100 in 0.1% Normal Donkey Serum, 0.025% TritonX-100 (for the intracellular BACE2 localization) or just in 0.1% Normal Donkey Serum (for the plasma membrane BACE2 localization) in PBS. As a negative control cells were incubated with the fluorescent secondary antibody only.

Bio-Plex Assay

Phosphoprotein Detection: Phosphorylation of 8 different proteins was determined using the Bio-Plex™ suspension array system kit (Bio-Rad Laboratories, Reinach, Switzerland). Cells were washed and frozen once at -80°C to facilitate cell lysis, resuspended in 150 µl lysis buffer (provided in the kit) and protein concentration was determined by the Bradford method. 45 µg proteins were used to detect and quantify the phospho-proteins according to the manufacturer's guidelines. Phosphorylation was normalized to protein content. The phosphorylation of the following proteins was analyzed: cAMP response element-binding protein (CREB Ser^{133}), Insulin receptor substrate 1 (IRS-1 Ser^{636}/Ser^{639}), 70 kDa ribosomal S6 kinase (p70 S6 Kinase Thr^{421}/Ser^{424}), extracellular signal-regulated kinases (ERK1/2 Thr^{202}/Tyr^{204}, Thr^{183}/Tyr^{185}), p38 mitogen-activated protein kinase (p38 MAPK Thr^{180}/Tyr^{182}), glycogen synthase kinase 3 α/β (GSK-3 α/β Ser^{21}/Ser^{9}), c-jun oncogene (c-jun Ser^{63}) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α Ser^{32}/Ser^{36}).

Cytokines Detection: IL-1β, IL-6 and TNF-α cytokines levels were quantified in the plasma of ApoE-/- and ApoE BACE2-Tg mice was determined using the Bio-Plex™ suspension array system kit according to the manufacturer's guidelines.

Generation of BACE2 Overexpressing Mice and Dietary Treatment

The studies and animal facilities were approved by the "Kommission für Tierversuche des Kantons Zürich" (Switzerland). Housing and experimental procedures were in accordance with the Swiss animal protection law. The pCAG_WT BACE2 plasmid containing full length sequence of human BACE2 under the control of the CAG promoter was propagated in

Escherichia coli DH5α and the minigene was excised with *Not*I. The fragments were purified from a 1% agarose gel with Qiaquick extraction kit (Qiagen, Basel, Switzerland) and processed following the manufacturer's recommendations. The transgenetic techniques were performed at the Institute of Laboratory Animal Science of the University of Zurich under SPF conditions. Nuclear injections into fertilized C57BL/6J oocytes were carried out by conventional methods [34, 35].

Transgenic founder were identified by PCR using a CAG- promoter specific primer and a BACE2- specific primer (CAG and BACE2 primers, **Supplementary Table 4)**. One founder, C57BL/6J Tg(cag-AsgN)1372^{zbz}, was identified and crossed with C57BL/6J ApoE-deficient mice to generate C57BL/6J ApoE$^{-/-}$ tgBACE2$^{+/-}$ mice. Mice were bred always with C57BL/6J mice to exclude positioning effects due to transgene insertion. Genotyping of the mice was performed using the same primer combination as for the identification of the founder animals. Following primers were used for the ApoE locus: oIMR0180, oIMR0181 and oIMR0182 (**Supplementary Table 4**) [36]. These primers generate a 155 bp band for the WT locus and a 245 bp band for the targeted locus. If the animals are heterozygous both products are amplified [36].

Healthy male mice (C57BL/6J ApoE$^{-/-}$ and C57BL/6J ApoE$^{-/-}$ BACE2-Tg) were housed at the institutional animal facilities on a 12:12-h light-dark cycle. Animals had free access to food and water. In one treatment group mice were fed for 10 weeks with control diet (Kliba Nafag 3430, Kaiseraugst, Switzerland) whereas in the other treatment group mice were fed with standard chow for 10 weeks followed by a high fat/cholesterol diet (Research Diet D12108) for 15 weeks. At the end of the treatment, both groups were anesthetized (20 mg/kg body weight Xylazine, 100 mg/kg body weight Ketamine and 3.0 mg/kg body weight Acepromazine in 0.9% NaCl) and exsanguinated via cardiac puncture. Organs for RNA expression were cleaned in RNA-later (Qiagen, Basel, Switzerland) and snap-frozen in liquid nitrogen and stored at -80°C. Blood was centrifuged at 5,900 g at 4°C for 15 min and plasma was stored at -80°C.

Glucose Tolerance Tests and Lipid Measurements

In the week of the experiment mice were starved overnight, weighed and fasting glucose levels were measured, followed by glucose tolerance test (GTT) as described [37].

Levels of plasma cholesterol, triglycerides and free fatty acids were determined using colorimetric assays (Roche Applied Science). Separation of lipoproteins was performed by FPLC as described [38].

Quantification of Atherosclerosis

Mice were sacrificed and the aorta was dissected, cleaned from the perivascular fat under a microscope, cut open longitudinally and pinned onto a black-colored silicon plate. The whole aorta was fixed in 4% PFA at 4°C overnight followed by oil red O staining overnight. The pinned aortas were washed twice and stored in PBS at 4°C. Pictures of stained aortas were taken using a Nikon (coolpix 2200) digital camera and atherosclerotic plaques area were quantified using the Image J (NIH Image) software program and calculated in percentage of total area.

Statistical Analysis

All statistical analyses were performed using the GraphPad or StatView software. Since the data of each experiment were normally distributed (D'Agostino and Pearson omnibus normality test), comparisons of group means were performed by two-tailed paired Student's *t*-Test (to compare the means of two matched groups), by two-tailed unpaired Student's *t*-Test followed by Welch's correction (to compare the means of two unmatched groups) or by one-way ANOVA followed by Bonferroni post-hoc test (to compare the means of three or more groups). Statistical significance was accepted at P values < 0.05. Data are expressed as means±SEM. For time courses, area under the curve (AUC) was calculated using the GraphPad software.

RESULTS

Characterization of BACE2-Mediated Protease Activity

BACE2 is a transmembrane protein of 57 kDa, containing a protease domain with two catalytic aspartate (D) residues **(Fig. 1a)**. To analyze protease activity of human wild-type (WT) and protease-inactivated (D110A and D303A, MUT) BACE2 proteins an *in vitro* fluorescent-based activity assay was performed using lysates from HEK293T cells transiently transfected with WT, MUT BACE2 or control vector (CTL) and a FRET-competent substrate representing the peptide sequence of the alternative α-cleavage site of APP. In lysates of WT but not the MUT BACE2 expressing cells an enhanced proteolytic activity was measured compared to CTL cells as quantified by integration of cleavage activity over time (area under the curve: 2336±541 vs. 818±77.8, P=0.006, **Fig. 1b**). Similar expression levels of the constructs were confirmed by Western blot (**Fig. 1c**). Only in cells expressing WT BACE2 an additional band of 54 kDa was visible (**Fig. 1c**) indicating that prodomain processing of BACE2 is dependent on an intrinsic functional protease domain confirming data suggesting an autoproteolytic activation mechanism [22, 39].

Subcellular Localization of BACE2 in Human Vascular Smooth Muscle Cells

To investigate subcellular distribution of human BACE2, VSMCs were fractionated. Endogenous (CTL) and stably overexpressed WT and MUT BACE2 were detected exclusively in the triton-insoluble membrane fraction as analyzed by Western blot (**Fig. 1d**) indicating that subcellular distribution is independent of protease activity. Purity of the fractions was confirmed with fraction-specific antibodies: α-tubulin for cytoplasmic fraction, the small G-protein $G_{\alpha q/11}$ for the triton-soluble and caveolin-1 for the triton-insoluble fraction (**Fig. 1d**).

To further characterize subcellular compartmentalization of endogenous BACE2, cells were immunostained for cellular compartment specific marker proteins and for BACE2 followed by confocal microscopy (**Fig. 1e**). BACE2 was partially co-localized with early endosomal compartments, caveolae and the trans-Golgi network as visualized in yellow in the merged pictures. In the lysosomes no co-localization was detected (**Fig. 1e**).

Downregulation of BACE2 Gene and Protein Expression in VSMCs by Proliferative Stimuli

As expression of regulatory molecules may be affected in proliferating VSMCs during atherogenesis [40], the effect of proliferative stimuli on BACE2 gene and protein expression levels was investigated in human VSMCs, as an *in vitro* model of atherogenesis. Exposure of VSMCs to PDGF-BB, LDL or FCS downregulated steady-state BACE2 mRNA expression levels starting at 3 h with a further decrease till 24 h post stimulation (**Fig. 2a**, $P<0.05$ vs. CTL). Exposure to FCS for 24 h had the strongest effect resulting in downregulation of 74.2±2.4% compared to control cells. Similarly, BACE2 protein expression decreased after exposure to FCS and PDGF-BB as shown by immunoblot (**Fig. 2b**). Immunofluorescence analysis also revealed a time-dependent decrease of BACE2 protein after FCS stimulation, which was already detectable after 5 min (**Fig. 2c**). These results suggest an inhibitory effect of proliferative stimuli such as FCS, LDL and PDGF-BB on BACE2 mRNA and protein expression levels.

As BACE2 is localized in the plasma membrane and in intracellular membranes ([39, 41] and **Fig. 1e**), we analyzed whether BACE2 protein expression was differently affected in the plasma membrane compared with intracellular compartments after stimulation with FCS. BACE2 expression in the plasma membrane (unpermeabilized cells) and total cellular expression (permeabilized cells) was quantified by measurement of cellular mean fluorescence intensity (MFI) using an antibody against the N-terminal extracellular part of BACE2 and flow cytometry. In unstimulated cells, plasma membrane-localized BACE2 was 5-fold lower compared with total BACE2 protein expression and remained unchanged after FCS stimulation (open bars, **Fig. 2d, upper left and lower panel**). Total amount of BACE2 protein (black bars) was reduced after 10 min of FCS stimulation (-27±6.5%, $P=0.017$) and further decreased after 30 min of treatment (-47.3±7.6%, $P=0.001$, **Fig. 2d, upper right and lower panel**). The rapid decrease of BACE2 protein expression observed after FCS stimulation suggested rapid degradation via proteasomal pathways [42]. To test this hypothesis cells were preincubated with 2 different proteasome inhibitors followed by FCS stimulation. The FCS-dependent decrease of BACE2 protein amount was abolished in cells preincubated with proteasome inhibitors (**Fig. 2e**) indicating a critical role of the proteasome in BACE2 protein degradation, a mechanism, which was previously demonstrated for its homologue BACE1 [43].

Crucial Role of Protease Activity of BACE2 on Cell Proliferation

Since a decrease of BACE2 protein after incubation with proliferative stimuli is accompanied with cell proliferation, we tested whether BACE2 has a regulatory role in cell growth. Indeed, overexpression of WT BACE2 in VSMCs resulted in significant decrease in cell proliferation as measured by [^{3}H]-thymidine incorporation after FCS stimulation compared to control cells (-49±8.2%, *P*=0.0009, **Fig. 3a**). Overexpression of MUT BACE2 in VSMCs had no effect on cell proliferation compared to control cells (92.2±9.3% vs. 100%, n.s, **Fig. 3a**) indicating that the growth inhibitory effect of BACE2 in human VSMCs depends on its intrinsic proteolytic activity. This finding was further confirmed in FLTRs cell line in which overexpression of WT, but not of MUT BACE2 resulted in decrease of cell proliferation as determined by cell counting (-50.3±2.4%, *P*=0.0005 vs. CTL, **Fig. 3b**). In order to further confirm the role of BACE2 in VSMCs proliferation, endogenous BACE2 gene expression was downregulated using gene-specific siRNA oligonucleotides (**Supplementary Fig. 3**). Cell proliferation in BACE2 siRNA-transfected cells was 80±20.5% and 72±20.1% increased after stimulation with 2% or 10% FCS compared to control cells, respectively (*P*=0.042 for 2% FCS and, *P*=0.037 for 10% FCS, **Fig. 3c**). Similarly, pretreatment of the cells with a β-secretase inhibitor, which has been shown to also inhibit BACE2 enyzmatic activity [44], increased FCS-induced cell proliferation (43±13%, *P*=0.002, **Fig. 3d**). Taken together, we could demonstrate that an active protease domain of BACE2 is essential to inhibit cell proliferation of smooth muscle cells.

No Direct Role of Soluble Fragments Generated by BACE2 in Inhibition of Cell Proliferation

Inhibitory signals can be transmitted via soluble ligands which are synthesized as trans membrane precursor proteins and released by proteolysis from the cell into the cell culture supernatant [45]. To investigate the possible involvement of this mechanism in inhibition of cell proliferation by BACE2 “supernatant transfer” experiments were conducted. Supernatants from neither the WT nor MUT BACE2 overexpressing cells had an effect on FCS-induced proliferation of VSMCs (**Supplementary Fig. 4a**). Next we analyzed whether an intracellular soluble fragment which could be generated by γ-secretase cleavage, subsequent to BACE2 proteolytic cleavage, is involved in inhibition of cell growth. Pretreatment with the γ secretase inhibitor had also no effect on cell proliferation (99.03±3.7% vs. 100%, n.s, **Supplementary Fig. 4b**). Together, these data indicate that the growth-inhibiting factor/s generated by BACE2 is/are not released into the cell supernatant or cytoplasm.

BACE2 Inhibits Pro-Inflammatory Signaling Pathways

To investigate different signaling pathways possibly contributing to the BACE2-mediated anti-proliferatory effect, phosphorylation kinetics of key molecules shown to play a role in the regulation of VSMCs cell proliferation were determined using bioplex array system. A decrease in phosphorylation, was observed in WT BACE2 overexpressing cells for c-jun (-41±11.3%, *P*=0.022, **Fig. 4a**) and IκB-α (-31±5.3%, *P*=0.01, **Fig. 4b**) after 30 min of FCS stimulation compared to control cells. In the other proteins investigated (CREB, GSK-3 α/β, IRS-1, p70 S6 Kinase, ERK1/2 and p38 MAPK) no effect of BACE2 overexpression on phosphorylation kinetics was observed (**Supplementary Fig. 5**).

Next we determined gene expression levels of the pro-inflammatory molecules IL-6, IL-1β and cox2 in untreated and FCS stimulated cells.

In untreated cells expression of IL-6, IL-1β and cox2 was at a low level and not affected by BACE2 overexpression. Stimulation with FCS resulted in an increased gene expression of at least 2-fold and BACE2 overexpression resulted in a 27±10% (*P*=0.031, **Fig. 4c)** and 26±10% (*P*=0.039, **Fig. 4c)** decrease in expression of IL-6 and cox2 compared to CTL cells, respectively. IL-1β was not affected by BACE2 overexpression. This result indicates that decreased phosphorylation of c-jun and IκB may result in decreased expression of the pro-inflammatory genes IL-6 and cox2 in VSMCs.

BACE2 Overexpression Also Decreases pro-Inflammatory Gene Expression and Additionally Atherosclerotic Lesions in ApoE$^{-/-}$ Mice

To study the functional role of BACE2 in an *in vivo* model of atherogenesis, we generated a mouse expressing a human WT BACE2 transgene under the control of a chicken actin promotor allowing ubiquitous expression of the gene. Heterozygote mice were used for experiments. Steady state mRNA expression levels of BACE2 were 3-fold higher in aortas of ApoE$^{-/-}$ BACE2-Tg mice compared to ApoE$^{-/-}$ control mice (**Supplementary Fig. 1b**). To investigate whether in an *in vivo* experimental setting expression of pro-inflammatory genes is also affected by BACE2 overexpression, steady state mRNA expression levels of VCAM-1, IL-6, IL-1β, cox2 and TNF-α were analyzed in aortic tissue by real-time PCR. In the first treatment group, mice were fed with control diet and sacrificed at 10 weeks of age. Expression levels of the inflammatory genes IL-6, IL-1β and TNF-α in the thoracic aorta were at a low level, whereas cox2 was expressed about 10-fold higher (**Fig. 5a**) and no differences amongst the two mice strains were observed. At this stage no atherosclerotic plaques were

observed in the aorta of both ApoE$^{-/-}$ and ApoE$^{-/-}$ BACE2-Tg mice ([24], and data not shown). In the second treatment group, mice were subjected to an additional high-fat, cholesterol diet for 15 weeks at the age of 10 weeks. All genes analyzed were 2 to 10-fold higher expressed compared to the control diet group (**Fig. 5a, b**). In thoracic aorta of ApoE$^{-/-}$ BACE2-Tg mice expression of IL-1β was decreased by 56% and of cox2 by 53% compared to control ApoE$^{-/-}$ mice (**Fig. 5b**), whereas expression of IL-6 and TNF-α was similar across groups. In these mice atherosclerosis was quantified by measuring oil red O-stained areas representing lipid-rich, atherosclerotic plaques in longitudinally opened aortas. As shown in **Fig. 5c** and **5d** overexpression of WT BACE2 decreased total lesion area by 28% (23.8±1.4% in ApoE$^{-/-}$ mice to 17.2±1.7% lesion areas in ApoE$^{-/-}$ BACE2 Tg mice, P=0.006). Atherosclerotic plaques were slightly more abundant in the abdominal compared to thoracic region of the aorta, but in both parts plaque areas were significantly decreased in the ApoE$^{-/-}$ BACE2-Tg mice (abdominal aorta: 18.8±2.3% vs. 27.3±2.3%, P=0.014; thoracic aorta: 15.8±1.9% vs. 21.4±1.6%, P=0.032, **Fig. 5d**). Together, these data suggest that BACE2 decreases the formation of atherosclerotic lesion and inhibits the expression of pro-inflammatory proteins in the aorta.

Decreased Plasma Levels of Lipids and Pro-Inflammatory Cytokines in the Presence of BACE2 Transgene

Levels of pro-inflammatory cytokines in the blood plasma were analyzed. IL-1β levels were downregulated by 44% (P=0.037) and a trend towards downregulation for IL-6 (-33%, P=0.17) and TNF–α (-40%, P=0.06) was observed suggesting a systemic anti-inflammatory effect of BACE2 transgene expression (**Fig. 6a**). In line with these data, plasma levels of cholesterol (-11%, P= 0.0006), triglycerides (-41%, P= 0.0001), and free fatty acids (-25%, P= 0.016) were also downregulated in this group (**Fig. 6b**). Lipoprotein profiles as analyzed by FPLC indicated less abundancy of VLDL and LDL in the plasma of ApoE$^{-/-}$ BACE2-Tg mice (-41%, **Fig. 6c**). No difference between groups was observed in regard to body weight, relative glucose tolerance and plasma insulin levels (**Supplementary Table 5**).

DISCUSSION

The present study demonstrates for the first time a role of BACE2 in atherogenesis and in the regulation of human VSMCs cell proliferation. In these cells, which are importantly involved in atherosclerosis development [5], we detected BACE2 in the plasma membrane and throughout all secretory membrane compartments including Golgi apparatus/TGN and early endosomes, a distribution pattern which was similarly demonstrated in neuronal cells and other cell types [22, 39, 41]. Endocytic organelles constitute of a complex network of subcellular compartments, which are actively involved in transport to and uptake of membrane-associated signaling molecules from the plasma membrane thereby controlling signal transduction. Intriguingly, our data also indicate that BACE2 is co-localized with caveolin-1 and is found in the triton-insoluble cell compartment, indicating that BACE2 resides in lipid rafts as demonstrated for its homologue BACE1 [46, 47]. Lipid rafts are small platforms of tightly packed lipid bilayer composed of sphingolipids and cholesterol coordinating and regulating a variety of signaling processes [48]. This suggests that BACE2 may play a role in raft-originating intracellular signaling pathways.

To characterize the potential role of BACE2 in atherosclerosis, functional aspects of the disease such as cell proliferation and plaques development were analyzed. We speculated that in healthy condition, BACE2 may act as a gate keeper to maintain VSMCs in a quiescent, contractile phenotype, whereas in diseased condition, BACE2 may be downregulated allowing phenotype switching to a proliferative, synthetic phenotype characteristic for vascular disorders like atherosclerosis. Frequently, downregulation/degradation of proteins is mediated via proteasomal pathways which have been shown to be overstimulated in atherosclerosis [49]. Since our data showed decreased expression of BACE2 in atherosclerotic tissues *in vivo* (unpublished observation) as well as a FCS-dependent degradation of BACE2 in VSMCs via proteasomal pathways *in vitro*, it is possible that decreased BACE2 expression is a result of overstimulated proteasomal pathways. This may be a mechanism to further promote the atherosclerotic process via increased VSMCs proliferation. A similar expressional regulation has been demonstrated for hyperplasia suppressor gene/mitofusin-2 which inhibits serum-mediated VSMCs proliferation and is downregulated during atherogenesis [40].

In order to investigate how BACE2 could mediate inhibition of cell proliferation we tested two possible mechanisms. First, being the antiproliferative effect of BACE2 secretase-dependent, we investigated whether the proteolytic activity of BACE2 was responsible for the

release of ligands into the supernatant that are involved in growth inhibition. This mechanism, known as ectodomain shedding, is already demonstrated for many ligands such as PDGF, EGF and TNF-α [45]. However, supernatants from BACE2 overexpressing cells had no additional effect on VSMCs proliferation indicating that the growth-inhibiting factor/s is/are unlikely to be released into the cell supernatant. Secondly, we tested whether γ-secretase cleavage, subsequent to BACE2 proteolytic cleavage, was involved in inhibition of cell growth. Rationale for this hypothesis was the instance of plasma membrane-bound Notch which transmits signals to the nucleus by γ-secretase-dependent release of a intracellular fragment regulating in that way gene transcription [50]. BACE2 cleaves APP preferentially at an alternative α-secretase site, resulting in soluble APP (sAPP) and a C-terminal part (APP CTF) which remains bound to the membrane. This membrane-bound APP CTF is further processed by a γ-secretase, generating a small peptide (p3), which is secreted, and a C-terminal fragment (CTFγ), which is released into the cytoplasm [41, 51]. Due to the fact that γ-secretase inhibition had no effect on cell proliferation it appears unlikely that CTFγ of APP or proteolytically cleaved cytoplasmic domains of so far unknown substrates of BACE2 are involved in growth inhibition. Together, our data indicate that proteolytic activity of BACE2 is not involved in generation of intra- or extracellular fragments which directly block cell proliferation.

Inflammation is often associated with proliferation of VSMCs which promotes atherosclerosis development [3]. Analyzing intracellular signaling pathways after FCS stimulation we found that VSMCs overexpressing BACE2 showed reduced phosphorylation of the c-jun, an integral part of the dimeric transcription factor AP-1, and IκBα, an inhibitor of the transcription factor NF-κB, indicating a reduced activation of the pro-inflammatory transcription factors AP-1 and NF-κB [15, 52]. Indeed, we observed after stimulation with FCS a low but significant decrease in gene expression of pro-inflammatory molecules such as interleukin 6 (IL-6) and cyclooxygenase 2 (cox2) in cells overexpressing BACE2. Both genes are known to be under transcriptional control of NF-κB [53, 54] and AP-1 [54, 55] in VSMCs. Cox2 is an inducible enzyme responsible for the synthesis of prostanoids (such as prostaglandins, prostacyclins and thromboxanes) and is known to be involved in inflammatory processes including atherosclerosis [56]. IL-6 is a pro-inflammatory cytokine, which has an important impact on cell proliferation and atherosclerotic plaques development [57, 58].

Looking at inflammation in our mouse model of atherosclerosis, we could show that mice which were free of atherosclerotic lesions expressed very low levels of pro-inflammatory cytokines in the aorta independent of BACE2 protein amount. However, upon high-fat diet treatment, the analyzed cytokines were expressed at a higher level, and, in support to our *in*

vitro data, transgenic mice showed more then 50% reduced expression of cox2 and of the pro-inflammatory growth-promoting cytokine interleukin 1β (IL-1β) [59, 60]. IL-1β concentration was also found to be strongly reduced in the plasma of the same mice. Interestingly, binding of IL-1β to its receptor mediates activation of both NF-κB and AP-1 transcription factors [61] leading to transcription of many genes responsible for proliferation and inflammation. IL-1β binds to IL-1 receptor type I (IL-1RI), which is also known to be expressed in VSMCs [60] and to IL-1RII. Proteolytic cleavage of the extracellular part of IL-RI [62] and IL-RII [18] has been demonstrated, whereof IL-1RII can be cleaved by BACE2 [18]. Moreover, it has been shown that antagonizing IL-1β signaling, suppresses atherosclerotic lesion development in ApoE-deficient mice [63]. Our data, showing a reduced expression of pro-inflammatory genes in VSMCs overexpressing BACE2 and in aorta of $ApoE^{-/-}$, BACE2-Tg mice suggest a connection between BACE2-mediated reduction of cell proliferation and inflammation. Currently, we are analyzing whether IL-1 receptor type I is cleaved by BACE2 in VSMCs, resulting in "receptor decapitation" and therefore in the downregulation of the downstream signaling pathways.

In support to our gene expression data from aortic tissue, Fernandez-Hernando and co-worker also showed using $ApoE^{-/-}$ and $ApoE^{-/-}$, $Akt^{-/-}$ mice, that differences in atherosclerosis-related gene expression depend on high-fat diet [64]. This indicates that besides modification of the mouse genotype a strong atherogenic stimulus such as high-fat/cholesterol diet is needed to identify expressional differences in atherosclerosis-related genes. Our *in vivo* data are compatible with the notion that BACE2 expression is strongly involved in the downregulation of pro-inflammatory gene expression. *In vitro*, on the contrary, the effects were rather small suggesting that either different cell types (i.e. macrophages or endothelial cells) or a distinct VSMC phenotype (closer resembling highly activated VSMCs found in atherosclerotic lesions) contribute to the decreased expression of pro-inflammatory cytokines in the plasma and in the aorta of BACE2 transgenic mice.

An elevated level of LDL cholesterol is one of the most important risk factor for the development of atherosclerosis [2]. As BACE2 transgene expression lowered the level of the LDL cholesterol fraction by more than 40% it is highly likely that this dramatic decrease contributes to the decreased atherosclerotic lesion size in the aorta. Further studies are warranted to analyze the underlying cellular and molecular mechanism by which BACE2 regulates cholesterol and lipid metabolism.

In conclusion, our data suggest for the first time a role of BACE2 in the regulation of proliferation and inflammation in VSMCs *in vitro*. Additionally, BACE2 appears to be

involved in the inhibition of atherosclerotic plaques development by regulating plasma levels of pro-inflammatory cytokines and lipids *in vivo*. Interestingly, it has been shown that patients with trisomy 21 have a slower progression of atherosclerotic vascular changes compared to controls throughout the cardiovascular system [65]. This observation may be explained by a protective role of BACE2 due to a gene dosis effect as BACE2 is located on chromosome 21 at position 21q22.3 in human [17], which is the critical region of the Down syndrome (trisomy 21). Thus, the specific induction and activation of BACE2 could provide a novel therapeutic approach to decrease progression of atherosclerosis.

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Contributions:

MB and EH designed the study; MB, EH and RM projected the experiments; EH and RM wrote the manuscript; MB, EH and RM interpreted the results and performed statistical analysis; RM, EH, EA and AP performed *in vitro* experiments, EH, PC and RM generated transgenic mice; RM, EH, IB and MD performed mice experiments; CW measured plasma levels of cholesterol, lipids and insulin and performed FPLC analysis; AP measured metabolic parameters of the mice.

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Figure Legends

Figure 1: Characterization of BACE2 Constructs and Subcellular Localization of BACE2 in Human Vascular Smooth Muscle Cells

a. Schematic representation of the BACE2 protein structure from N- to C-terminus. Full length BACE2 consists of a Signal peptide- (SP), Pro- (PO), Protease-, Transmembrane- (TM) and Cytoplasmic- (CP) domain. The two essential aspartic (D) catalytic residues for the secretase activity are indicated.

b. Protease activity time course of membrane extracts of HEK 293T cells transiently transfected with wild-type (WT), mutant (MUT) BACE2 or control (CTL) constructs. Extracts were incubated with a FRET-competent APP-based peptide for the times indicated. Fluorescence intensity (FI) was recorded at 320 and 420 nm excitation and emission wavelengths. Data are means±SEM, n=3, **P*=0.006 vs. CTL, †*P*=0.005 vs. MUT.

c. HEK 293T cells transiently transfected with wild-type (WT), mutant (MUT) BACE2 or control (CTL) constructs were analyzed by Western blot with an antibody against BACE2.

d. Subcellular distribution of BACE2 was analyzed in human VSMCs retrovirally transduced with wild-type (WT), mutant (MUT) BACE2 or control (CTL) virus constructs. Cells were subjected to cell fractionation and Western blot analysis using antibodies against BACE2 and compartment-specific proteins (α-tubulin for cytoplasma, the small G-protein $G_{\alpha q/11}$ for tritron-soluble and calveolin-1 for triton-insoluble membranes).

e. Vesicular distribution of BACE2 was analyzed in VSMCs by confocal immunofluorescence microscopy using an antibody against BACE2 (red) together with antibodies against specific marker proteins of different cell compartments (green). Co-localization is visualized in yellow in the merged pictures. Early Endosomes were localized using EEA1 antibody, lysosomes using CD107a antibody, caveolae using Caveolin-1 antibody and TGN using syntaxin antibody. Scale bars indicate 20 μm.

Figure 2: Effect of Proliferative Stimuli on BACE2 Gene and Protein Expression in Human Vascular Smooth Muscle Cells

a. Time course of BACE2 steady state mRNA expression levels after stimulation with LDL (50 μg/ml), PDGF-BB (20 ng/ml), FCS (10%) or solvent control (CTL) determined by real-time PCR. Data are means±SEM. ΔC_T values were calculated from the gene of interest and the housekeeping gene β-actin, and were expressed as percentage of solvent control-treated cells at time-point 0 min (100%). n=4-6 per group, **P*<0.0001 vs. CTL.

b. Incubation of VSMCs with FCS (10%) or PDGF-BB (20 ng/ml) was terminated at the indicated time points and cell extracts were analyzed by Western blot with antibodies against BACE2 and α-Tubulin as a loading control. A representative experiment out of 3 is shown.
c. VSMCs were stimulated with FCS (10%) for the indicated time frames and cells were analyzed by immunofluorescence using an anti-BACE2 antibody and a TRITC-labelled secondary antibody. Two representative examples are shown. Scale bars indicate 20 μm.
d. BACE2 protein expression after FCS stimulation was assessed by flow cytometry in unpermeabilized (Unperm= plasma membrane localized BACE2) and permeabilized (Perm= total BACE2) VSMCs using an antibody against the N-terminal part of BACE2 and a FITC-conjugated secondary antibody. Upper panels: representative histograms are shown of cells stimulated for 0 (green), 10 (pink), and 30 min (blue) with FCS (10%), respectively. Purple filled histograms represent negative controls. Lower panel: BACE2 protein expression measured by flow cytometry. Bars represent means±SEM, n=3 per group, **P*=0.017 (10 min) and **P*=0.001 (30 min) vs. Perm unstimulated.
e. BACE2 protein expression in VSMCs after MG-132 (10 μmol/l) and Lactacystin (20 μmol/l) preincubation (6 h) and prior to 10% FCS stimulation (30 min) was assessed by flow cytometry in permeabilized cells. Bars represent means±SEM, n=4-7 per group, **P*=0.002 vs. unstimulated, †*P*=0.006 (MG) and †*P*=0.006 (LAC) vs. FCS.

Figure 3: Effect of BACE2 on Cell Proliferation in Primary Human Vascular Smooth Muscle Cells and in Fetal Human Aortic Smooth Muscle Cell Line

a. Cell proliferation was measured by [^{3}H]-thymidine incorporation assays in control (CTL), wild-type (WT), and mutant (MUT) BACE2 stably overexpressing VSMCs after incubation with 0.1 % FCS (unstimulated) or 10 % FCS for 24 h. Bars represent means±SEM and are expressed as percentage of FCS-stimulated CTL cells (100%), n=4 per group, **P*=0.0009 vs. CTL, †*P*=0.003 vs. MUT.
b. Cell proliferation was assessed in control (CTL), wild-type (WT), and mutant (MUT) BACE2 stably overexpressing FLTR cells by cell counting. Cells were counted immediately before starvation (white bars, day 0), after starvation for 3 days (grey bars, day 3) and after stimulation with 10% FCS for 2 additional days (black bars, day 5). Bars represent means±SEM, n=4 per group, **P*=0.0005 vs. CTL, †*P*=0.002 vs. MUT.
c. Cell proliferation was assessed in VSMCs transfected with BACE2 siRNA (filled bars) or scrambled siRNA (open bars) after stimulation with 2% or 10% FCS. Bars represent

means±SEM and are expressed as percentage of 10% FCS-stimulated scrambled cells (100%), n=4 per group, **P*=0.042 (2%) and **P*=0.037 (10%) vs. scrambled siRNA.

d. Cell proliferation was assessed in VSMCs after preincubation (30 min) with a β-secretase inhibitor, prior to 24 h 10% FCS stimulation. Bars represent means±SEM and are expressed as percentage of 10% FCS-stimulated cells (100%), n=6 per group, **P*=0.002 vs. FCS stimulation alone.

Figure 4: Effects of BACE2 on Signaling Pathways Involved in Inflammation in Human Vascular Smooth Muscle Cells

Protein phosphorylation of c-Jun (**a**) and IKB-α (**b**) was assessed using Bioplex protein array system in control (CTL, open symbols) and wild-type (WT, filled symbols) BACE2 stably overexpressing human VSMCs after 10% FCS stimulation at the indicated time points. Phosphorylation was normalized to protein content. In the left panels the time course of protein phosphorylation is shown after stimulation with 10% FCS and expressed as percentage of $CTL^{0\ min}$. In the right panels the different phosphorylation status at time point 30 min are expressed as percentage of $CTL^{30\ min}$. Bars represent means±SEM, n=4-5 per group, (**a**) c-jun **P*=0.022 and (**b**) IKB-α **P*=0.01 vs. CTL.

c. Steady state mRNA expression levels of IL-6, IL-1β and cox2 were determined in control (open bars) and wild-type BACE2 overexpressing cells (black bars) using real-time PCR. Cells were left untreated (left panel) or FCS stimulated (right panel). Data are means±SEM. ΔC_T values were calculated from gene of interest and housekeeping gene β-actin and are expressed as percentage of control cells stimulated with FCS (100%). n=4 per group, IL-6 **P*=0.031 and cox2 **P*=0.039 vs. CTL.

Figure 5: Effects of BACE2 Overexpression on Atherosclerosis Development and on the Expression of Pro-Inflammatory Genes in the Aorta of $ApoE^{-/-}$ Mice

a./b. Steady state mRNA expression levels of IL-1, IL-1β, cox2 and TNF-α were analyzed by real-time PCR in the thoracic aorta of $ApoE^{-/-}$ (open bars) and $ApoE^{-/-}$ BACE2-Tg (black bars) mice after control diet (**a**) or high fat/cholesterol (**b**) diet. Data are means±SEM. ΔC_T values were calculated from gene of interest and housekeeping gene β-actin; n=2-4 per group.

c. Representative atherosclerotic plaques staining (red colour) of longitudinally opened aortas (en face analysis) using Oil red O staining in $ApoE^{-/-}$ and $ApoE^{-/-}$ BACE2-Tg mice after high fat/cholesterol diet.

d. Quantification of atherosclerotic lesion area in total, thoracic and abdominal aorta in ApoE$^{-/-}$ and ApoE$^{-/-}$ BACE2-Tg mice. Data represent means±SEM and are expressed as percentage of thoracic, abdominal, or total aorta area, respectively, ApoE$^{-/-}$ (n=27), ApoE$^{-/-}$ BACE2-Tg (n=13), **P*=0.006 (total aorta), **P*=0.031 (thoracic aorta) and **P*=0.014 (abdominal aorta) vs. ApoE$^{-/-}$ mice.

Figure 6: BACE2-Tg Expression Reduces Plasma Levels of Pro-Inflammatory Cytokines and Lipids after High-Fat/Cholesterol Diet

a./b. Plasma levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (**a**; n=9 per group.) cholesterol and lipids (**b**; n=10 per group) were quantified in fasted ApoE$^{-/-}$ (open bars) and ApoE$^{-/-}$ BACE2-Tg mice (filled bars). CHL: cholesterol, TG: triglycerides, FFA: free fatty acids. Data represent means±SEM, **(a)** IL-1β **P*=0.037 vs. ApoE$^{-/-}$ and (b) CHL **P*=0.0006, TG **P*=0.0001, FFA **P*=0.016 vs. ApoE$^{-/-}$.

c. Lipoprotein profiles measured by FPLC analysis in fasted ApoE$^{-/-}$ (open bars) and ApoE$^{-/-}$ BACE2-Tg mice (filled bars) mice. Data represent lipoprotein profile of pooled plasma from 10 animals.

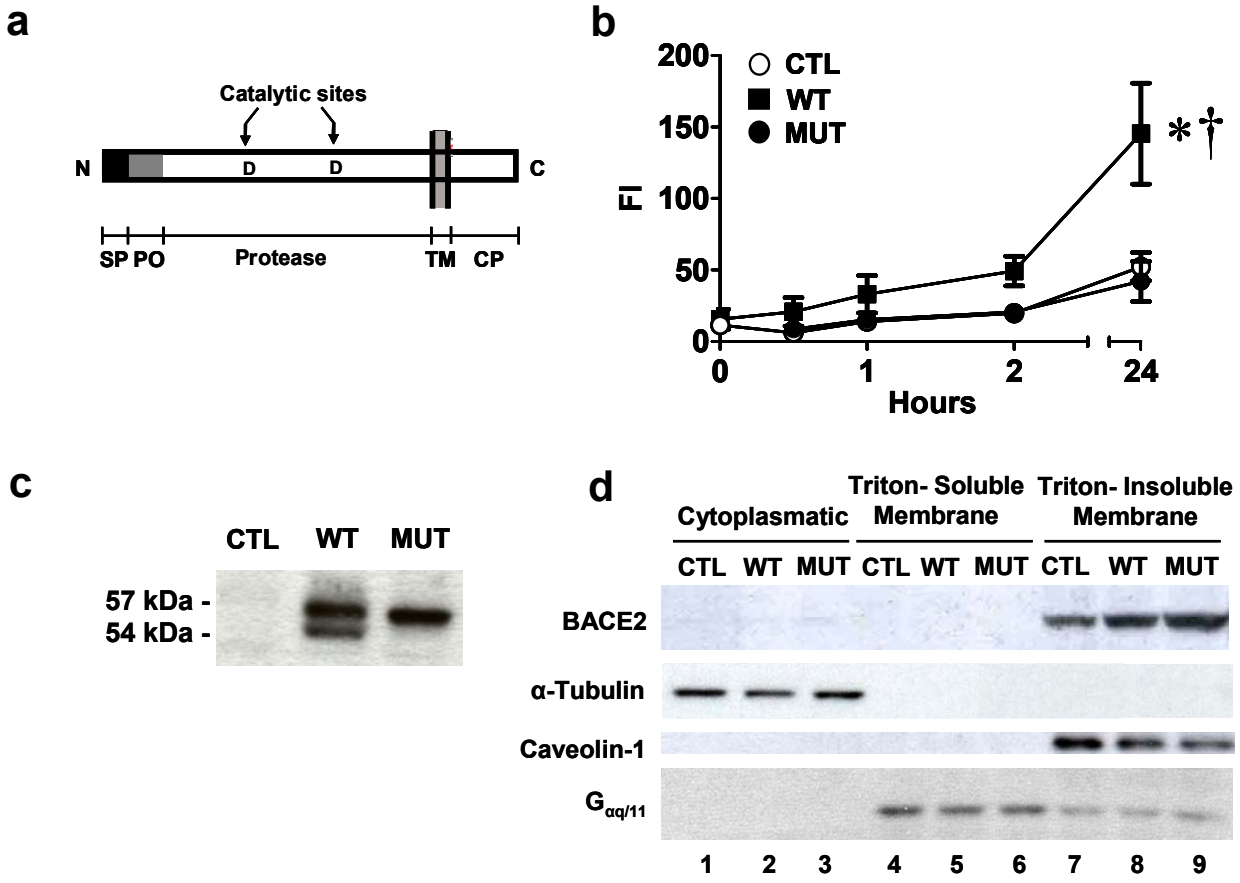
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Figure 1

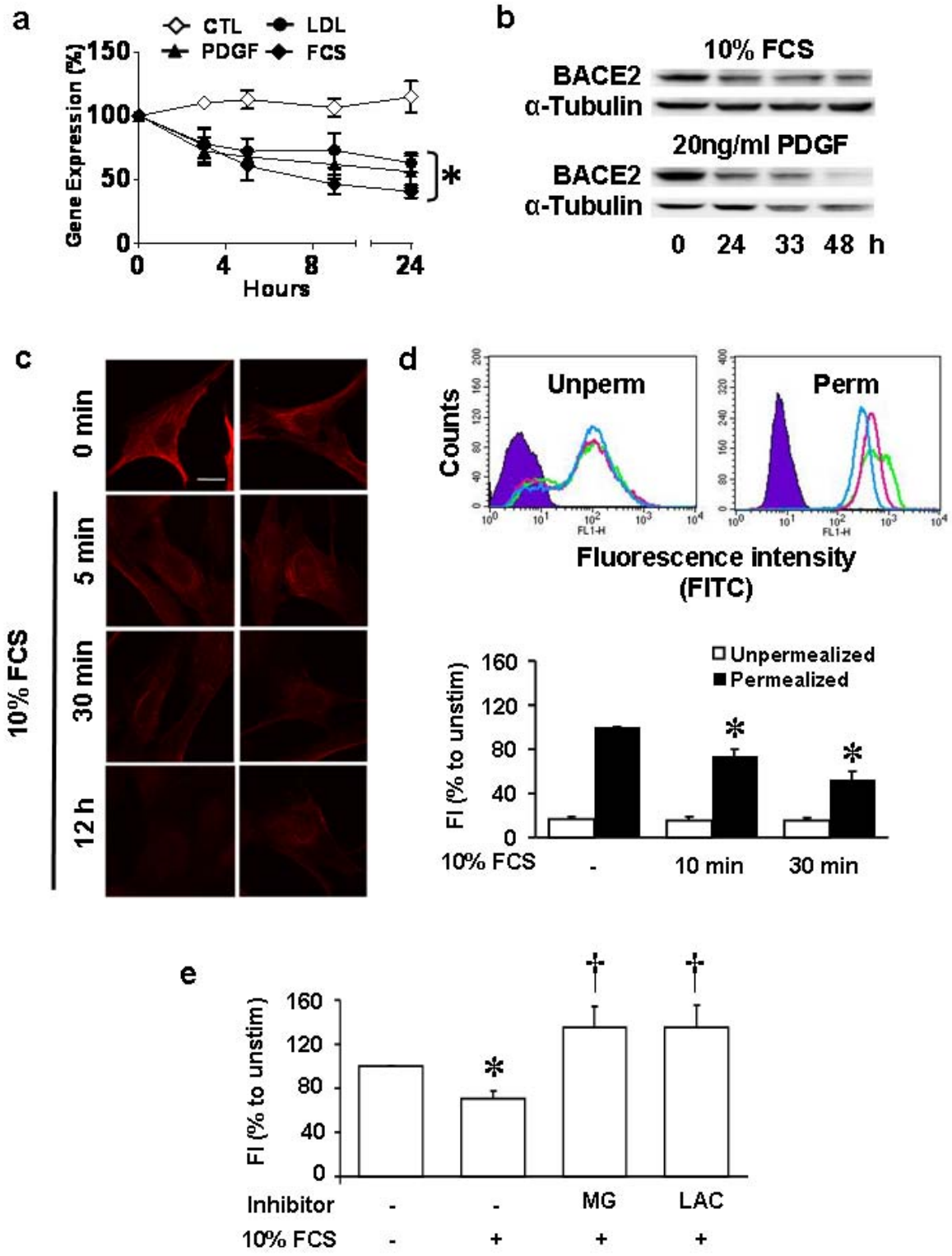
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Figure 2

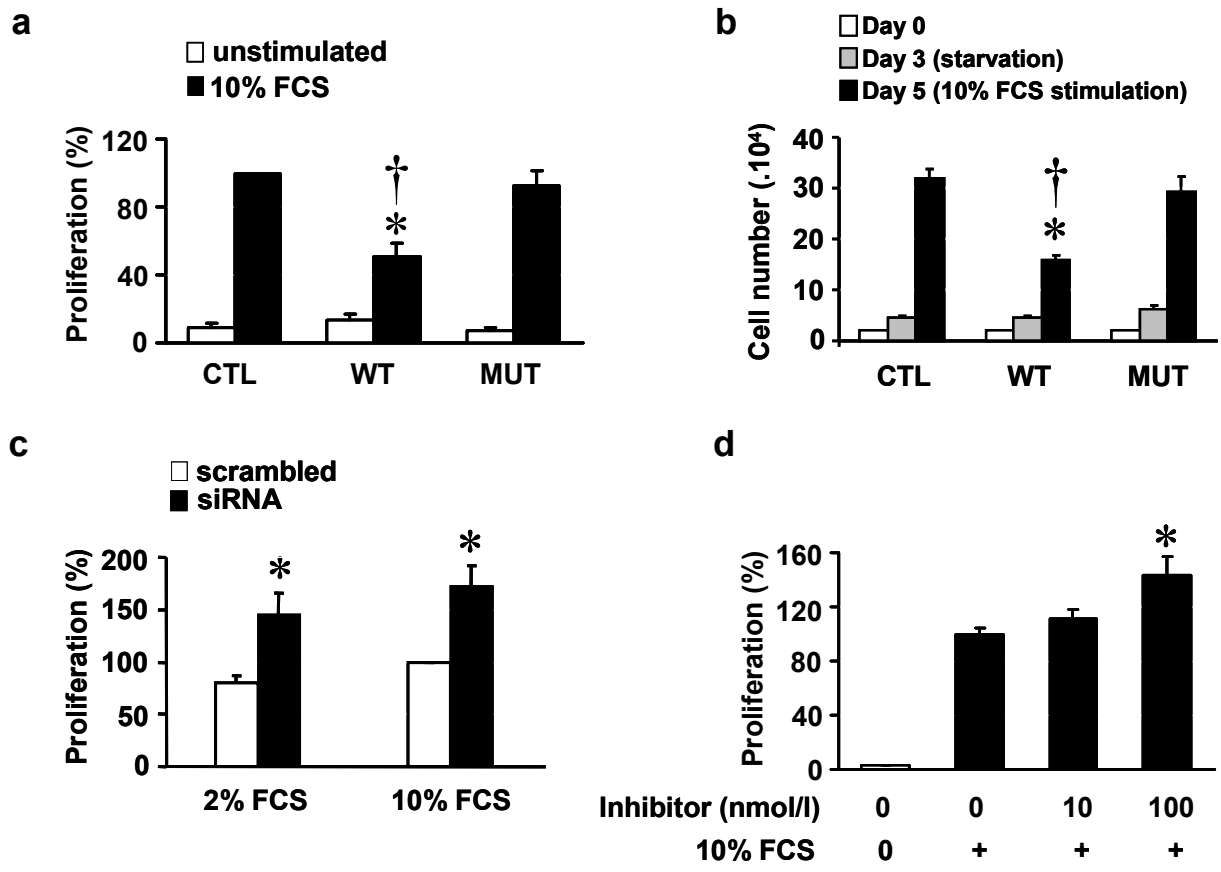
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Figure 3

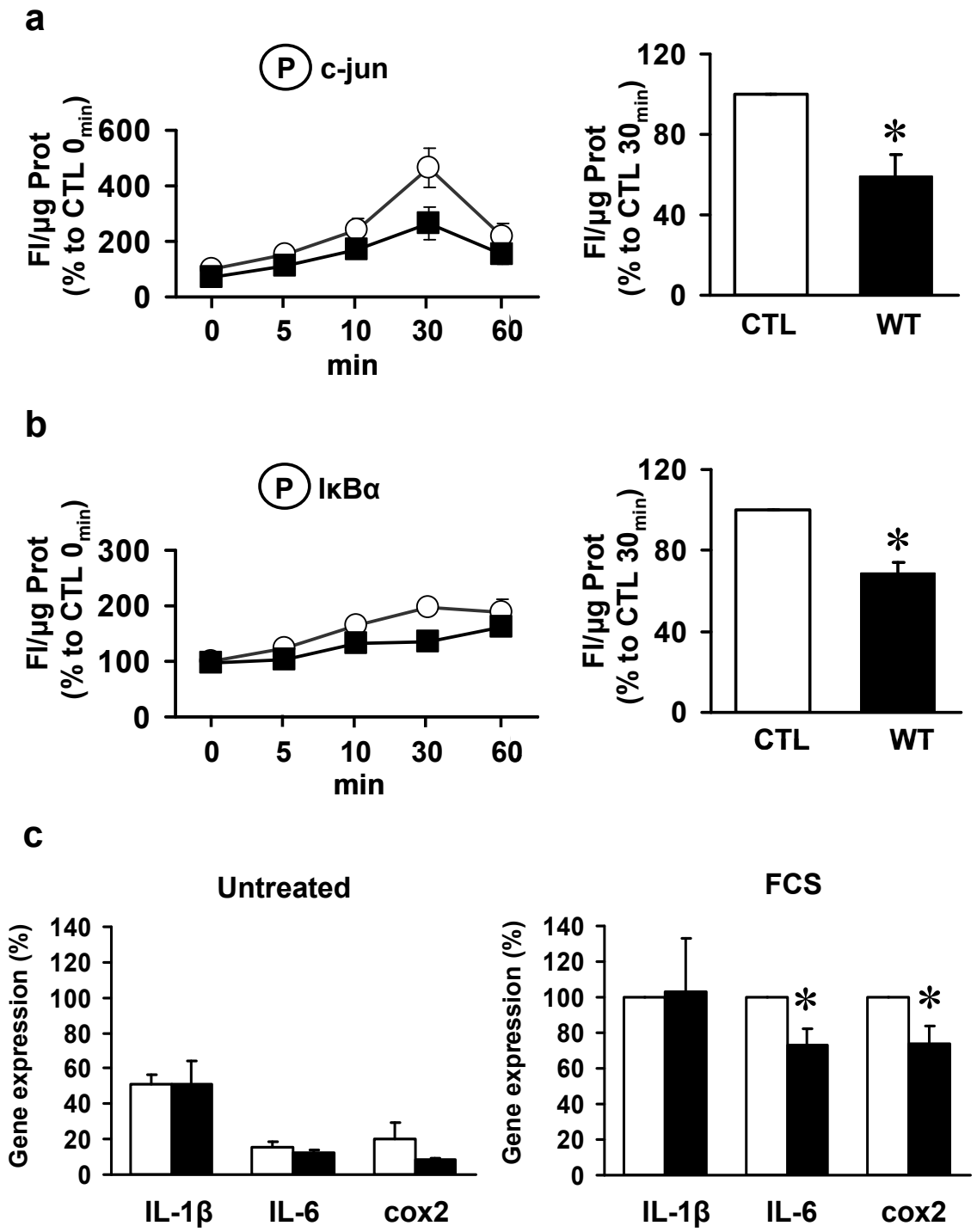
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Figure 4

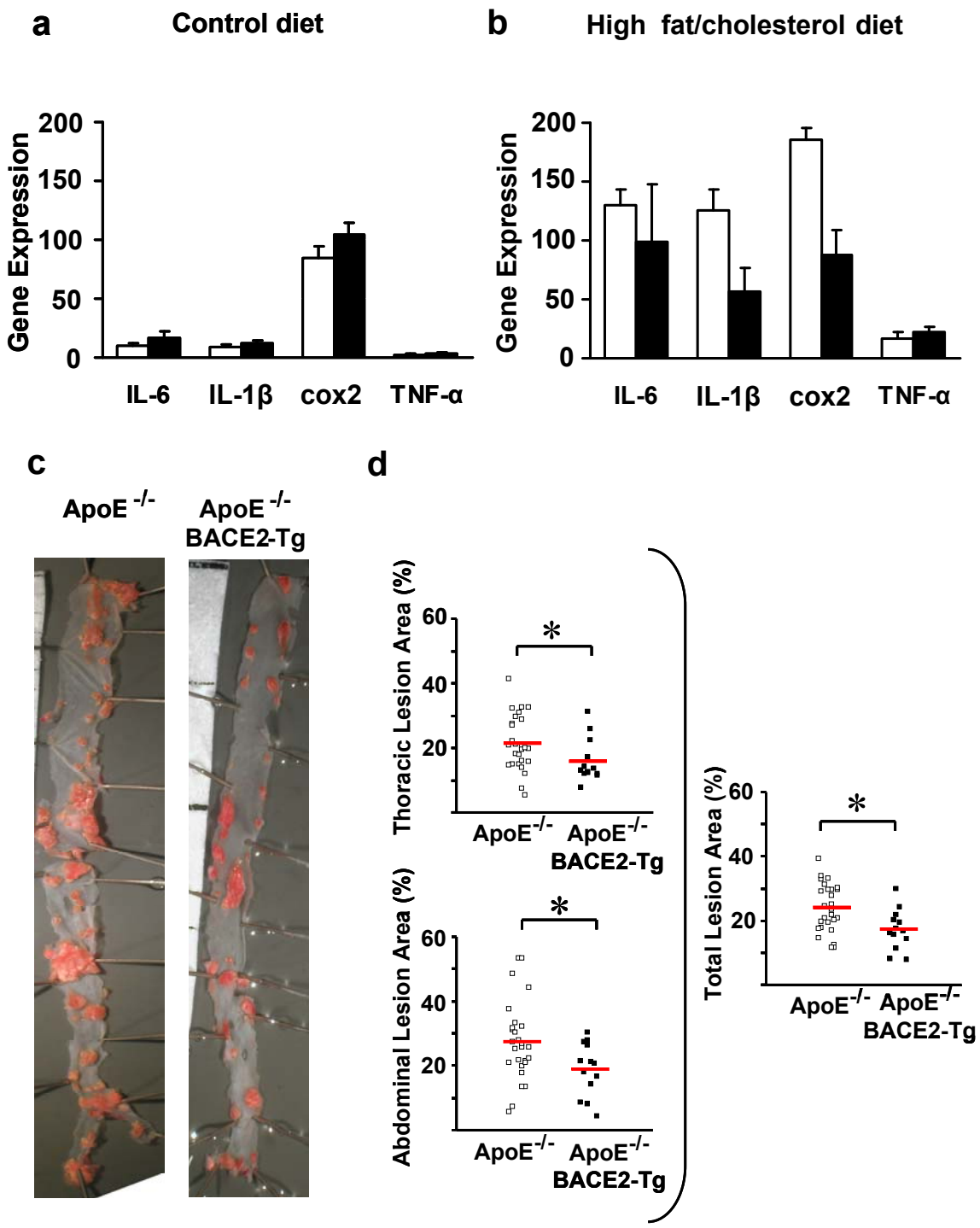
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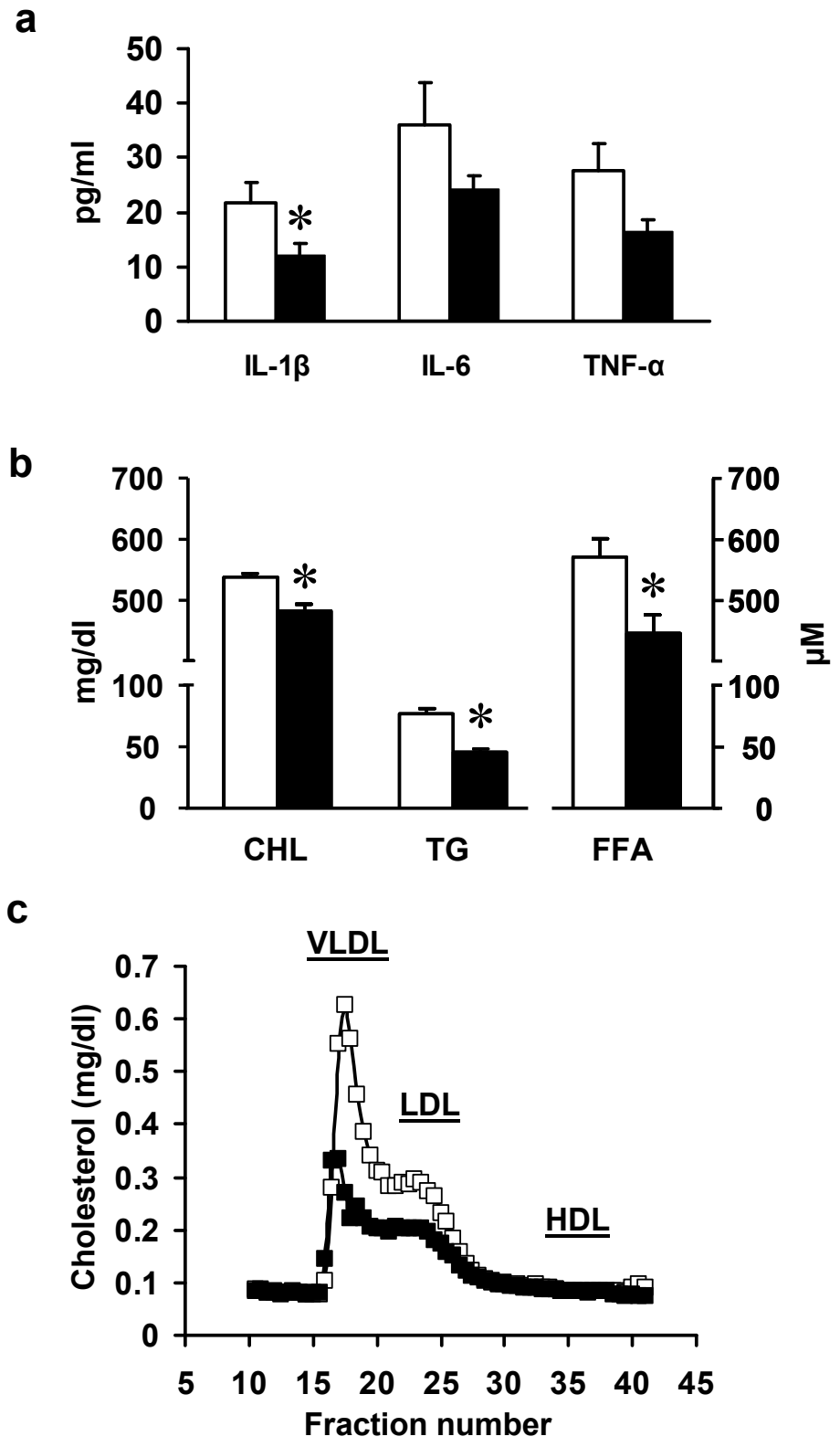
Figure 5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600
500
100
50
0
mg/dl
μM
*
*
*
CHL
TG
FFA
c
VLDL
LDL
HDL
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cholesterol (mg/dl)
5
10
15
20
25
30
35
40
45
Fraction number

Figure 6

Supplementary Information

1. Material and Methods

Supplementary Table 1: Oligonucleotides Used for Cloning

Name	Forward primer Reverse primer
forD110A	5'-GCTACAGATTCTCGTTGCTACTGGAAGCAGTAACTTTGCCG-3'
revD110A	5'-CGGCAAAGTTACTGCTTCCAGTAGCAACGAGAATCTGTAGC-3'
forD303A	5'- GCCATCGTGGCTAGTGGCACCACGCTGC-3'
revD303A	5'-GCAGCGTGGTGCCACTAGCCACGATGGC-3'
forXbaI	5'-CAATGCTCTAGAGCCACCATGGGCGCACTGGCCCGG-3'
revBamHI	5'-CAATGCGGATCCTCAGCGGGTTTAAACGGGCCC-3'
forSalI	5'-ACGGGTCGACGCCACC**ATG**GGCGCACTGGCCCGGGCGCTGCTGC-3'
revHindIII	5'-AAGCAAGCTTGTCATTTCCAGCGATGTCTGACCAGAGAGG-3'
forLinker	5'-AATTCGCCGTCGACTATAAGCTTCGCG-3'
revLinker	5'-AATTCGCGAAGCTTATAGTCGACGGCG-3'

Supplementary Table 2: Primers Used for Amplification of Human cDNA

Gene accession number	Forward primer Reverse primer	Product size (bp)
BACE2 AF178532	5`-GAT TCTCGT TGACACTGGAAG CA-3` 5`-CAAAGCCCTTGGAGCGGTATG-3`	113
IL-6 NM_000600	5'-TTCGGTCCAGTTGCCTTC-3' 5'-TCGTTCTGAAGAGGTGAGTG-3'	132
IL-1β NM_000576	5'-TTACAGTGGCAATGAGGATGAC-3' 5'-AGTGGTGGTCGGAGATTCG-3'	128
Cox2 AY462100	5'-CCCTGAGCATCTACGGTTTG-3' 5'-CATCGCATACTCTGTTGTGTTC-3'	107
TNF-α NM_000594	5'-AAGGACACCATGAGCACTG-3' 5'-AGAAGAGGCTGAGGAACAAG-3'	118

Supplementary Table 3: Primers Used for Amplification of Mouse cDNA

Gene accession number	Forward primer Reverse primer	Product size (bp)
BACE2 NM_019517	5'-GCAGAGAGTATAACGCAGACAAGG-3' 5'-CATCAAACACCTTCTGGGGCAG-3'	78
IL-6 NM_031168	5'-CCATCCAGTTGCCTTCTT G-3' 5'-AATTAAGCCTCCGACTTGTG-3'	139
IL-1β NM_008361	5'-GCTTCAGGCAGGCAGTATC-3' 5'-AGGATGGGCTCTTCTTCAAAG-3'	133
Cox2 NM_011198	5'-GCGAGCTAAGAGCTTCAGG-3' 5'-GAAGAGCATCGCAGAGGTG-3'	121

Supplementary Table 4: Primers Used for the Genotyping of Mice

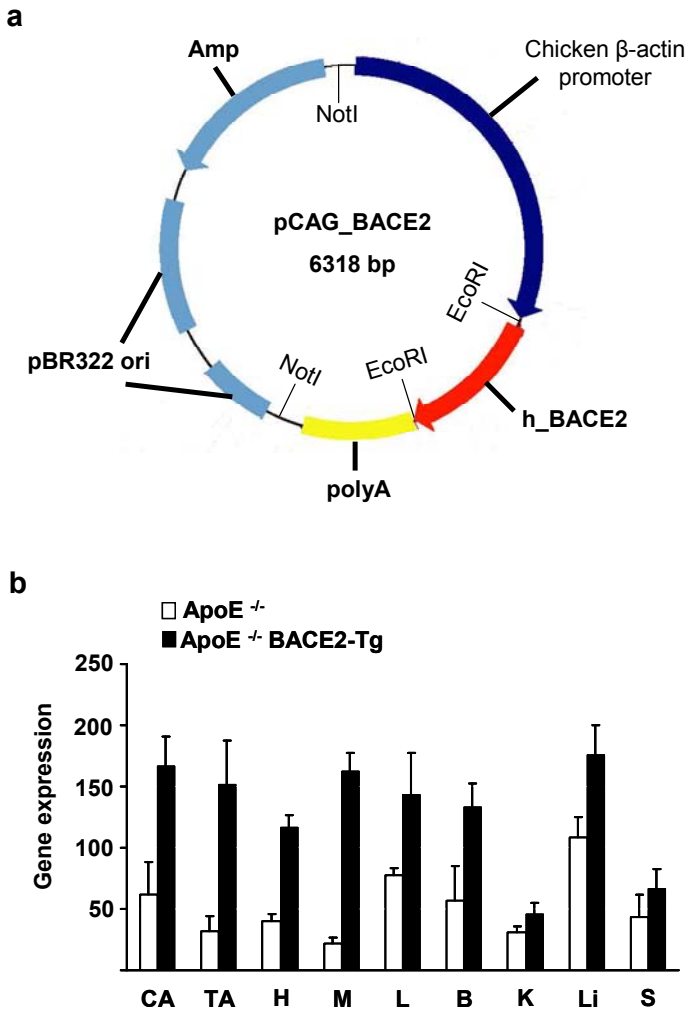
Name	Forward primer Reverse primer
CAG	5'-GGG TTC GGC TTC TGG CGT G-3'
BACE2	5'-TCCTGCCACGGCAAAGTT-3'
oIMR0180	5'-GCCTAGCCGAGGGAGAGCCG-3'
oIMR0181	5'- TGTGACTTGGGAGCTCTGCAGC-3'
oIMR0182	5'- GCCGCCCCGACTGCATCT -3'

Supplementary Table 5: Metabolic Parameters in ApoE$^{-/-}$ and ApoE$^{-/-}$ BACE2-Tg Mice After 15 Weeks of High-Fat Diet

	ApoE$^{-/-}$	ApoE$^{-/-}$ BACE2-Tg
Body weight (g)	28.8 ± 0.4 (28)	28.5 ± 0.5 (11)
Glucose (AUC)	1732.7 ± 59.4 (31)	1621.2 ± 80.3 (17)
Insulin (ng/ml)	0.343 ± 0.03 (10)	0.341 ± 0.02 (10)

Glucose tolerance tests were performed prior to sacrifice and body weight was measured immediately before experiments in ApoE$^{-/-}$ and ApoE$^{-/-}$ BACE2-Tg Mice. Glucose tolerance is presented as area under the curve (AUC) generated on the basis of glucose measurements from 0 to 120 min after intraperitonal glucose injection. Fasting insulin levels from both groups were determined after 15 weeks of high-fat diet feeding. Data represent means$\pm$SEM, n-numbers are indicated in brackets.

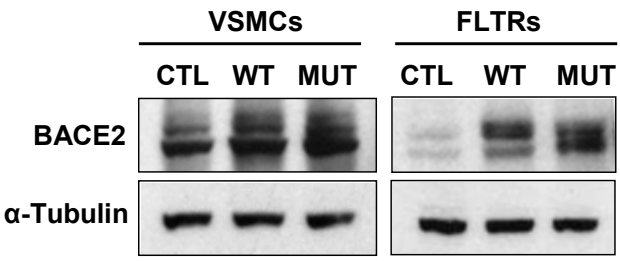
2. Supplementary Figures



Supplementary Figure 1: pCAG-BACE2 Mammalian Expression Vector and Overexpression of BACE2 in the Carotis and Aorta of Transgenic Mice

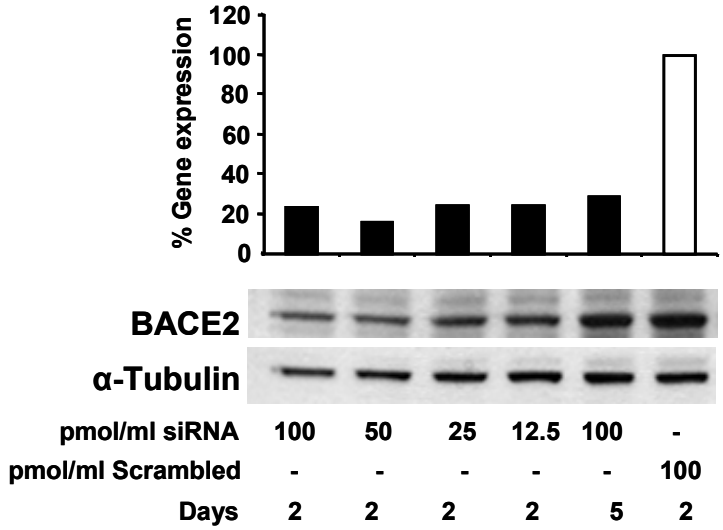
a. Schematic representation of the pCAG-BACE2 mammalian expression vector. The vector consists of the human BACE2 sequence (h_BACE2; red) controlled by the chicken β-actin promoter (blue) and followed by a polyA tail (yellow). The prokaryotic parts of the vector are represented in light blue and are needed for replication in bacterial cells (pBR322 ori and the ampicilin resistance gene).

b. Steady state mRNA expression levels of BACE2 in carotid artery (CA), thoracic aorta (TA), heart (H), muscle (M), lung (L), brain (B), kidney (K), liver (Li) and spleen (S) from ApoE$^{-/-}$ and ApoE$^{-/-}$ BACE2-Tg mice after high fat/cholesterol diet analyzed by real-time PCR. Data are means±SEM. ΔC_T values were calculated from gene of interest and housekeeping gene β-actin; n=3-4 per group.



Supplementary Figure 2: Overexpression of BACE2 in Fetal Human Aortic Smooth Muscle Cell Line (FLTRs) and in Human Vascular Smooth Muscle Cells (VSMCs)

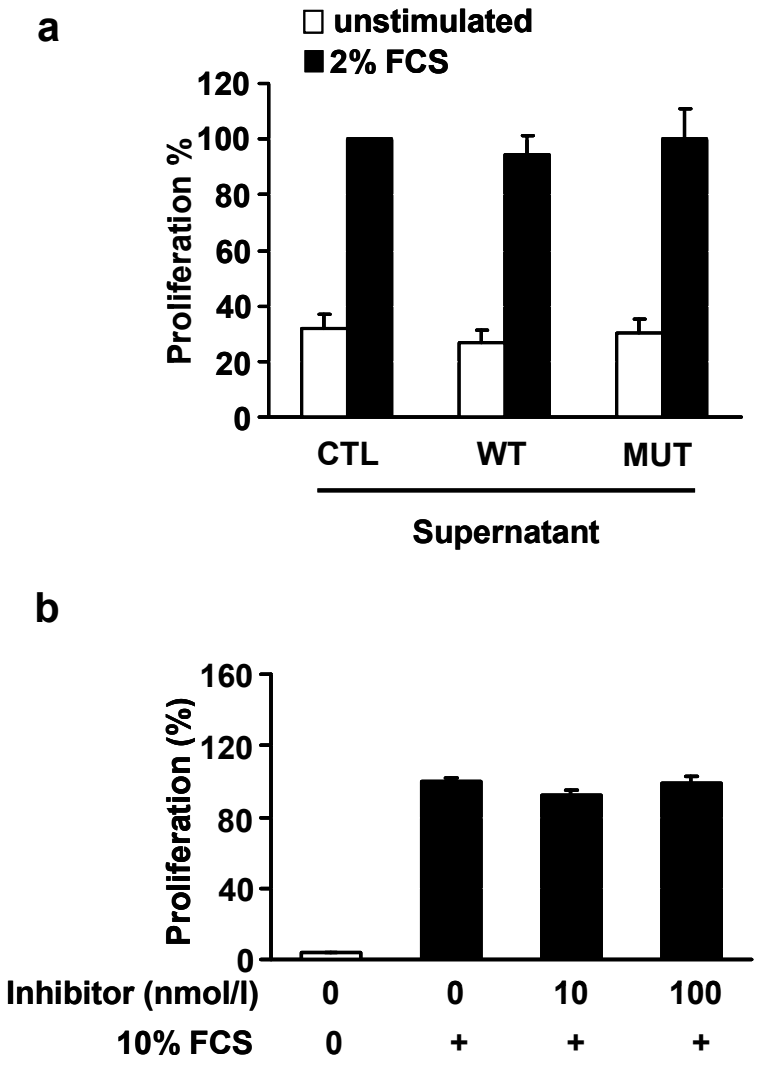
Cell extracts from VSMCs and FLTRs retrovirally transduced with control (CTL), wild-type (WT) or mutant (MUT) BACE2 expressing viruses, were analyzed by Western blot using antibodies against BACE2 and α-Tubulin as loading control.



Supplementary Figure 3: Downregulation of BACE2 Gene and Protein Expression after siRNA Transfection in Human Vascular Smooth Muscle Cells

Upper panel: BACE2 steady state mRNA expression levels were determined using real-time PCR in VSMCs after transfection with BACE2-specific (black bars) and scrambled (white bar) siRNA. ΔC_T values were calculated from gene of interest and housekeeping gene β-actin and are expressed as percentage of scrambled transfected cells (100%).

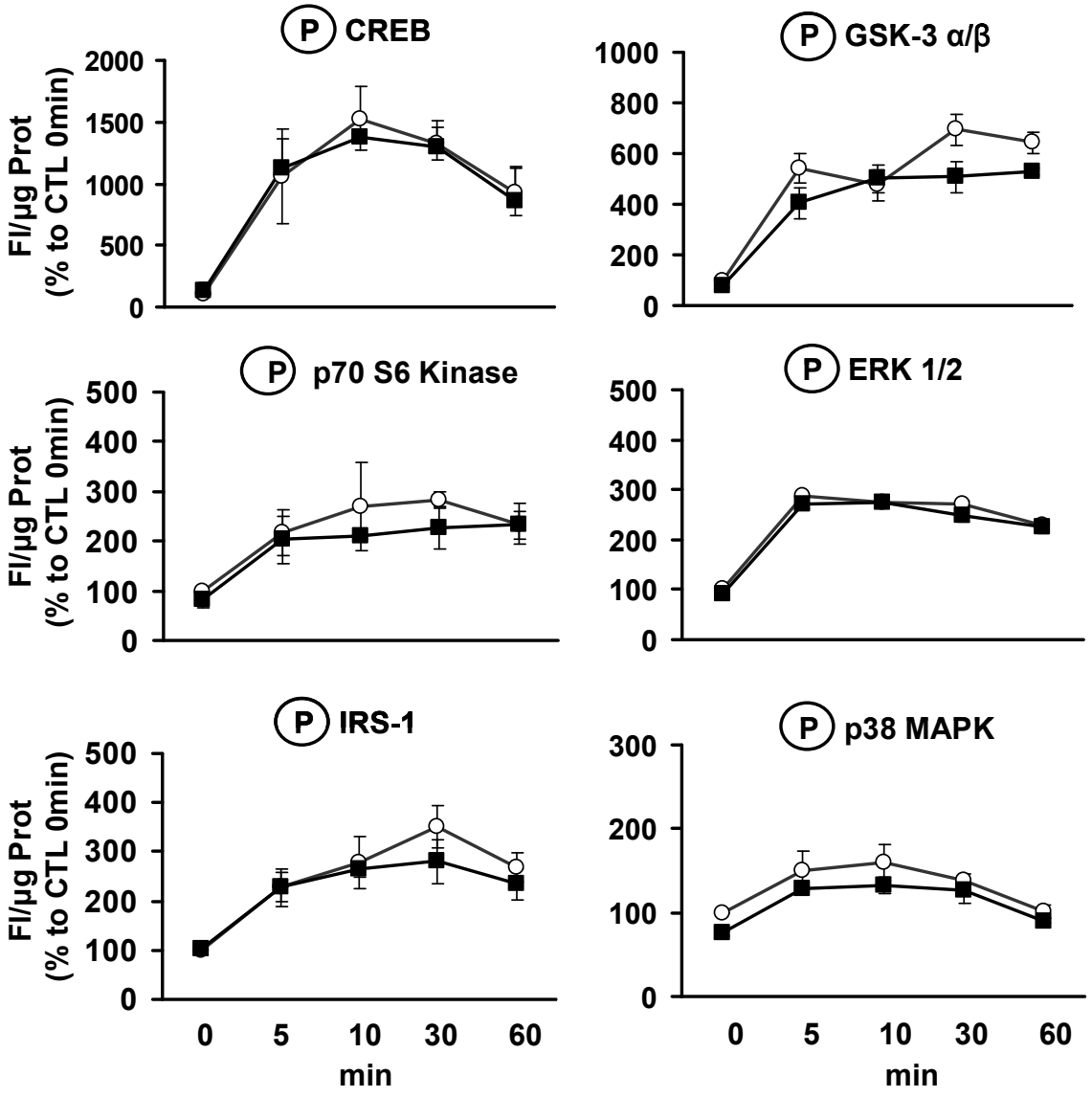
Lower panel: Cell extracts from BACE2-specific and scrambled siRNA transfected VSMCs were analyzed by Western Blot using antibodies against BACE2 and α-Tubulin as loading control. A representative experiment out of 3 is shown.



Supplementary Figure 4: No Direct Role of Soluble Fragments Generated by BACE2 in Inhibition of Cell Proliferation

a. Cell proliferation was assessed by [^{3}H]-thymidine incorporation assays in VSMCs treated with culture supernatants of control (CTL), wild-type (WT) or mutant (MUT) BACE2 stably overexpressing VSMCs. After supernatant transfer cells were stimulated with 2% FCS for 24 h (black bars) or kept unstimulated (white bars). Bars represent means±SEM and are expressed as percentage of 2% FCS-stimulated CTL cells (100%), n=5 per group.

b. Cell proliferation was assessed in VSMCs after preincubation (30 min) with a γ-secretase inhibitor, prior to 24 h 10% FCS stimulation. Bars represent means±SEM and are expressed as percentage of 10% FCS-stimulated cells (100%), n=3 per group.



Supplementary Figure 5: No Effect of BACE2 Overexpression on Phosphorylation of Signaling Molecules

CREB, GSK-3 α/β, p70 S6 Kinase, ERK1/2, IRS-1 and p38 MAPK phosphorylation was assessed using Bioplex protein array system in control (CTL, open symbols) and wild-type BACE2 (WT, filled symbols) stably overexpressing human VSMCs after 10% FCS stimulation for the indicated time frames. Phosphorylation was normalized to protein content. Bars represent means±SEM and are expressed as percentage of $CTL^{0\ min}$, n=4-5 per group.

3. EXPERIMENTS NOT INCLUDED IN THE MANUSCRIPT DRAFT

3.1 *In Vitro* Experiments

3.1.1 Introduction

In the manuscript draft presented in this thesis, a modulatory effect of BACE2 on VSMCs proliferation and on pro-inflammatory gene expression was observed. However, the mechanisms how BACE2 mediates these effects remain unclear. In this part of the study, some mechanistic hypotheses were tested in more detail.

3.1.2 Methods

Ion-Exchange Fractionation of Cell Supernatants

Cell supernatants were collected, filtered trough a 0.45 μm filter, and applied on HiTrap QFF ion-exchange columns (GE-Healthcare, Zurich, Switzerland). The fractions were eluted using increasing concentrations of NaCl (from 100 mM to 1 M) in 20 mM triethanolamine pH 8 according to the manufacturer's guidelines. Before adding the purified eluted fractions to VSMCs, the samples were desalted using PD-10 desalting columns (GE-Healthcare, Zurich, Switzerland).

Stimulation of VSMCs with Growth Factors

VSMCs (50% confluent) were starved for 24 h (0.1% FCS) followed by stimulation with hepatocyte growth factor (HGF, RELIATech, Braunschweig, Germany), growth hormone (GH, Alexis Biochemicals, Lausen, Switzerland), Insulin-like growth factor-1 (IGF-1, Sigma-Aldrich, Deisenhofen, Germany) or epidermal growth factor (EGF, Sigma-Aldrich, Deisenhofen, Germany) for 18 h, followed by (methyl-^{3}H)-thymidine incorporation assay as described in the manuscript draft.

Coomasie Blue Analysis of Cell Supernatant

Cell Supernatant from BACE2 (WT), BACE2 (MUT) overexpressing VSMCs and from CTL cells were collected and proteins were concentrated using Amicon Ultra-10K Centrifugal Filter Devices (Millipore, Zug, Switzerland). Protein concentration was measured by Bradford

method and equal amounts of proteins were separated by electrophoresis with 10% and 12% SDS/PAGE gels. The gels were stained with ProtoBlue Safe Colloidal Coomassie Blue (National Diagnostics, Basel, Switzerland) according to the manufacturer's guidelines. Differentially expressed proteins were excised from the gels and sequenced by mass spectrometry (performed by the Genomic center, University of Zurich, Zurich).

3.1.3 Results

Role of Cell Supernatant on Inhibition of Cell Growth

To investigate the potential role of soluble factors in the supernatant of BACE2 overexpressing cells for the inhibition of cell proliferation, "supernatant transfer" experiments were performed. Supernatants of BACE2 (WT), BACE2 (MUT) overexpressing VSMCs and of CTL cells were transferred onto untransduced VSMCs and cell proliferation experiments in the presence of 2% FCS were performed. As already presented in the **Supplemental Figure 4** of the manuscript draft, supernatants from neither the wild-type nor the secretase-inactivated BACE2 overexpressing cells had an effect on VSMCs proliferation, indicating that the growth-inhibiting factor/s is/are unlikely to be released into the supernatant. To exclude that the factor(s) was (were) missed due to too low concentration, supernatants were concentrated and fractionated by ion-exchange column. As shown in **Figure 11**, each fraction had different proliferative property but no difference between supernatant from CTL and WT cells was observed.

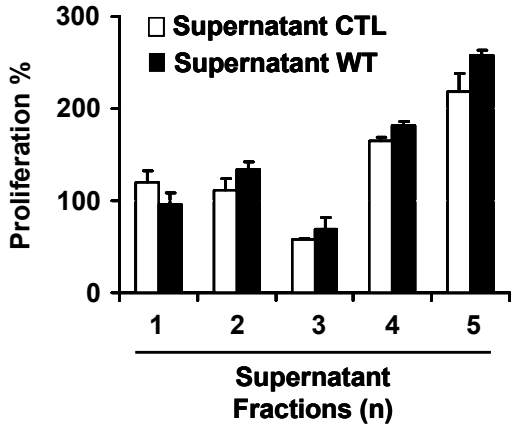


Figure 11: Effect of concentrated cell supernatant on cell proliferation in human VSMCs

Cell proliferation was assessed in untranduced VSMCs treaded with different fractions of cell supernatant from control (CTL) and wild-type BACE2 (WT) overexpressing VSMCs. Cell supernatants were fractionated by HiTrap QFF ion-exchange columns, followed by desalting using PD-10 columns. Bars represent means±SEM and are expressed as percentage of unstimulated cells, n=4 per group.

Effect of BACE2 Overexpression on Growth Factor-Dependent Cell Proliferation

Since a putative BACE2-dependent ectodomain shedding of a growth factor receptor was speculated, BACE2 (WT), BACE2 (MUT) overexpressing VSMCs and CTL cells were stimulated with different specific growth factors followed by cell proliferation assay. Basal proliferation was unchanged between groups (data not shown). Stimulation with HGF and GH had no effect on VSMC proliferation (**Figure 12**). Only after EGF and IGF-1 stimulation, a 2 to 3-fold increase in cell proliferation was observed, but BACE2 overexpression had no effect (**Figure 12**).

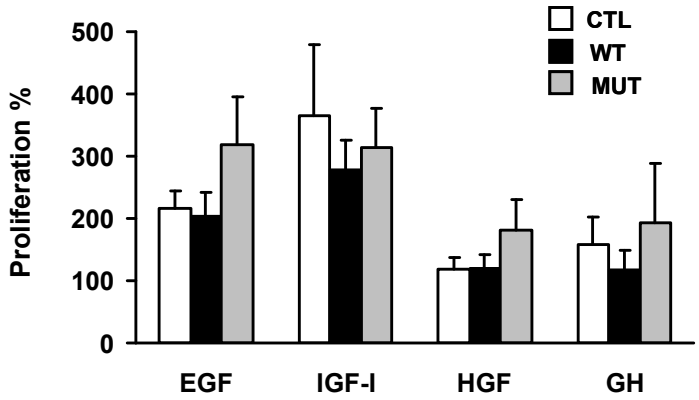


Figure 12: Effect of BACE2 on Growth Factors-Dependent Cell Proliferation in Human Vascular Smooth Muscle Cells. Cell proliferation was measured by [^{3}H]-thymidine incorporation assays in control (CTL), wild-type (WT), and mutant (MUT) BACE2 overexpressing VSMCs after 24 h incubation with insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), growth hormone (GH), epidermal growth factor (EGF) 100ng/ml each. Bars represent means$\pm$SEM and are expressed as percentage of unstimulated CTL cells (100%), n=4-6 per group.

Identification of Secreted and Shedded Proteins in VSMCs: Effect of BACE2

In a second approach supernatant from BACE2 (WT), BACE2 (MUT) overexpressing VSMCs and from CTL cells were collected. Proteins were concentrated, separated and analyzed by Coomassie Blue. Bands with differential intensity between groups were further investigated by mass spectrometry (**Figure 13**).

After protein staining with Coomassie Blue, no big differences between CTL, WT and MUT supernatants could be observed. However, three protein bands (a ~150 KDa, a ~90 KDa and a ~45 KDa band, red arrows, **Figure 13**), could be identified to be slightly more intense in the supernatant from BACE2(WT) overexpressing cells. After mass spectrometry analysis and with the help of a Mascot Search-software, proteins present in the three bands could be

identified. In the band running at 150 KDa (A, **Figure 13**) adipocyte enhancer binding protein 1, Isoform 1 of Fibronectin precursor and Collagen alpha-1(I) chain precursor were identified. In the 90 KDa band (B, **Figure 13**) heat shock protein 90-alpha/beta and Collagen alpha-2(I) were present. In the 45 KDa band (C, **Figure 13**) phosphoglycerate kinase 1 was identified.

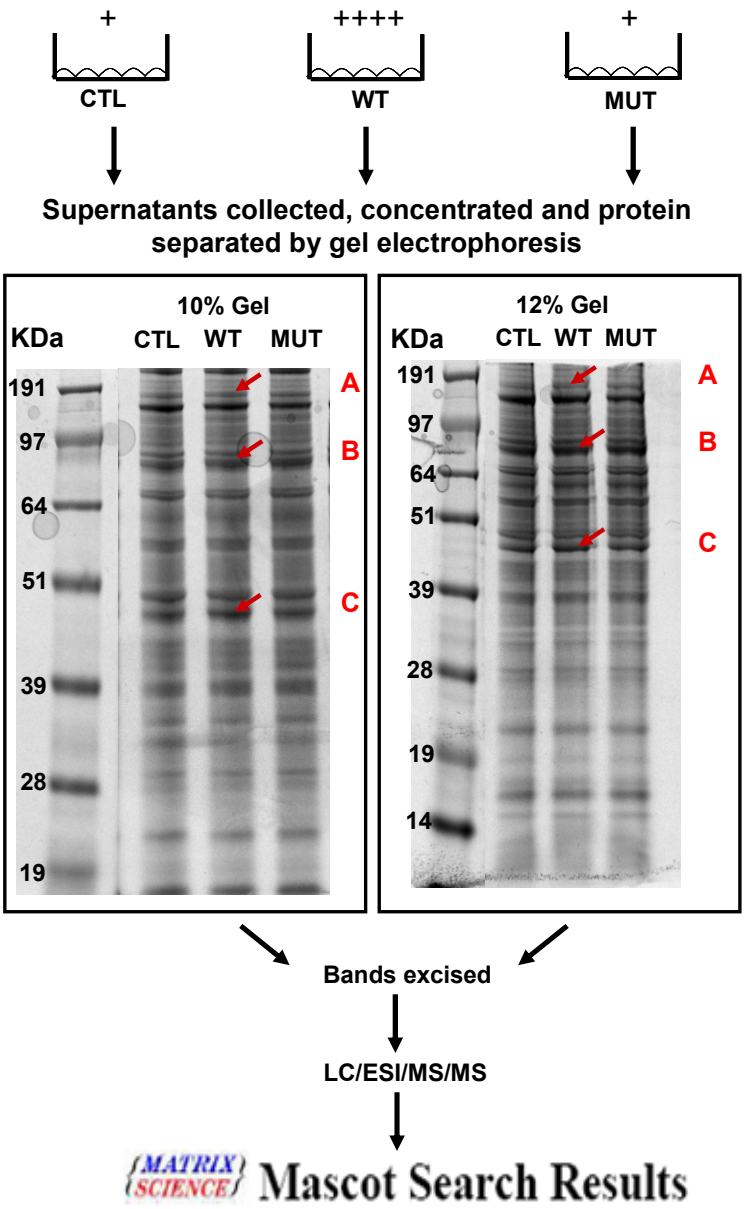


Figure 13: Schematic representation of the experimental procedure for the identification of proteins shedded into the supernatant. Supernatant from control (CTL), wild-type (WT), and mutant (MUT) BACE2 stably overexpressing VSMCs were collected and protein concentrated. After protein separation using 10% and 12% SDS/PAGE gels, proteins were stained by Coomassie Blue. Differentially expressed proteins (red arrows) were excised out of the gels, proteolitically digested and analyzed by liquid chromatography (LC), electrospray ionization (ESI) and mass spectrometry (MS). Results were analyzed by Mascot Search program.

3.1.4 Discussion

In this part of the study hypotheses regarding the potential mechanisms how BACE2 is modulating cell proliferation and pro-inflammatory gene expression in VSMCs were tested.
Supernatants from BACE2(WT), BACE2(MUT) and CTL VSMCs had no differential effect on VSMCs, neither in unconcentrated or concentrated form. These results suggest no role of BACE2-dependent shedded ligand/s in inhibition of cell proliferation.
The second hypothesis investigated in this part of the thesis was a putative BACE2-dependent post-translational downregulation of a growth receptor by ectodomain shedding. In a first approach, WT, MUT and CTL VSMCs were stimulated with different specific growth factors and proliferation was monitored. If BACE2 was responsible of cleaving the specific receptor for the investigated ligand, a reduced proliferation of WT cells could be expected. The ligands chosen are known to be involved in atherosclerosis and their specific receptors are already described to exist in soluble form. HGF and its receptor c-met are known to be expressed by VSMCs and have the capability to stimulate their proliferation and migration, two important feature of atherosclerosis development. C-met was also described to exist in a truncated soluble form, by the action of metalloprotease shedding [1]. Evidences that GH regulates VSMCs proliferation also exist [2, 3] and interestingly, it was shown that GH receptor undergoes metalloproteolytic cleavage resulting in receptor loss of function [4]. EGF has been demonstrated to be a potent mitogen in VSMCs and to be highly atherogenic [5] partly contributing to induction of VSMCs dedifferentitation [6]. The EGF receptor ErbB1 was also described to be proteolytic processed by metalloproteases [7]. Finally, binding of IGF-1 to its tyrosine kinase receptor activates a number of mitogenic pathways, leading to cell growth [8]. Also in VSMCs IGF-1 results in VSMCs migration and proliferation [9]. A protease-dependent ectodomain shedding was observed for IGF-1 receptor as well [10].
Surprisingly, stimulation with HGF, GH and EGF was not inducing proliferation of VSMCs. This may be explained by a technical limitation of the approach used in this experiment, or by a putative absence of the receptors for the chosen ligands. Passaging of cultured cells is known to induce deterioration of plasma membrane receptors [11]. The expression of cyclin GMP-dependent protein kinase receptor, for example, was described to decrease upon VSMC passaging [12]. For this reason, it may be possible that the VSMCs used for this experiment lost the receptors for the chosen growth factors during culture. To complete this analysis receptor expression of the ligands used in this study should be verified on protein level. Together, it should be noted that until further analysis are performed no conclusion could be drown.

In a second approach proteins present in the supernatant of CTL, WT and MUT VSMCs were separated by gel electrophoresis, and bands were visualized by Commassie Blue and analyzed using mass spectrometry to identify differences in shedded proteins. The differences identified by this approach were minimal, and the proteins assigned to the bands further analyzed by LC/ESI/MS/MS have probably no relevance for the mechanism hypothesized. Fibronectin [13] and Collagen [14] in fact are extracellular matrix protein, secreted by the cell mainly to fulfill structural function. Heat shock protein 90 was already demonstrated by Liao *et al.* to be secreted (and not shedded) by VSMCs under proliferative/stress conditions [15], and phosphoglycerate kinase 1, one of the enzyme involved in glycolysis, was also showed to be secreted [16]. Additionally, both proteins were identified at the molecular weight equivalent to the original full-size of the protein (90 and 45 KDa respectively). The same was observed for the ~175 KDa adipocyte-enhancer binding protein 1, a transcriptional repressor protein known to be involved in VSMC differentiation [17]; also in this case the protein was found at the expected molecular size as the full length protein. In conclusion, none of the proteins identified in this approach is a product of BACE2-dependent shedding, as all proteins analyzed were secreted. The assay chosen to test this hypothesis was probability not sensitive enough. A possible strategy to enhance sensitivity in this approach may be a pre-selection of the protein to be analyzed. Since the extracellular domains of shedded proteins are usually glycosylated [18], a good strategy may be to enrich and analyze only glycosylated peptides. This may eliminate from the analysis high abundant secreted proteins and therefore it may render small differences more visible. The use of 2D gel elecrophoresis instead of a normal one-dimensional SDS/PAGE gel and the labeling of amino acids with fluorescent dyes, may also help the identification of putative extracellular shedded product. The same was for instance done by Bech-Serra and co-workers [19] to analyze culture supernatant of ADAM17 overexpressing cells.

In conclusion, the experiments presented in this part of the thesis are still preliminary and do not allow any conclusion.

3.2 *In Vivo* Experiments

3.2.1 Introduction

In the manuscript draft presented in this thesis, I characterize the effect of BACE2 overexpression in ApoE$^{-/-}$ mice. In these mice the amount of atherosclerotic plaques and blood plasma concentrations of the pro-inflammatory cytokine IL-1β, cholesterol and lipids were reduced. It may be therefore interesting to characterize the effect of BACE2 secretase inactivation in the same animals. For this reason ApoE$^{-/-}$ BACE2$^{-/-}$ and control ApoE$^{-/-}$ mice were analyze in this part of the study.

3.2.2 Methods

Generation of C57BL/6J ApoE$^{-/-}$ BACE2$^{-/-}$ Mice Lacking BACE2 Catalytic Activity

BACE2 secretase-inactivated mice (BACE2$^{-/-}$) were kindly provided by Prof. Bart De Strooper and Prof. Paul Safting from the Center for Human Genetics, K.U Leuven and Flanders Interuniversity Institute for Biotechnology, Leuven, Belgium; and Christian Albrechts University, Kiel, Germany. These mice lack exon 6, containing one of the two aspartic catalytic residues indispensable for secretase activity. The mice were generated by Cre/lox P recombination technique introducing two lox P sites in the intron flanking exon 6 as described in [19].

BACE2$^{-/-}$ mice were back-crossed into C57BL/6J using speed congenics [20] and then crossed with C57BL/6J ApoE$^{-/-}$ to generate homozygous C57BL/6J ApoE$^{-/-}$ BACE2$^{-/-}$ mice. Genotyping of the mice for the BACE2 locus was performed using primers 5'-GCTATAGAGACCAAAGCCCACAAATCT-3' and 5'-GCCCGAATAACAAGAGCATCAC-3'.

For the ApoE locus oIMR0180, oIMR0181 and oIMR0182 primers (primers listed in the **Supplementary Table 4** of the manuscript draft) were used.

Housing and experimental procedure are described in the method section of the manuscript draft.

Metabolic Parameters, Cholesterol and Lipid Measurements

Body weights, fasting glucose levels, plasma level of cholesterol, triglycerides, free fatty acids and insulin, and the separation of plasma lipoproteins by FPLC were performed as described in the method section of the manuscript draft.

Quantification of Atherosclerosis

Atherosclerotic plaques of $ApoE^{-/-}$ and $ApoE^{-/-}$ $BACE2^{-/-}$ mice were visualized using oil red O staining as described in the method section of the manuscript draft.

Cytokines Detection

IL-1β, IL-6 and TNF-α cytokine levels were quantified in the plasma of $ApoE^{-/-}$ and $ApoE^{-/-}$ $BACE2^{-/-}$ mice using the Bio-Plex™ suspension array system kit according to the manufacturer's guidelines.

3.2.3 Results

Inactivation of BACE2 Secretase Activity Does Not Affect the Development of Atherosclerotic Lesions in the Aorta of ApoE$^{-/-}$ Mice

To further investigate the function of BACE2 in atherosclerosis development *in vivo*, we generated ApoE$^{-/-}$ mice lacking BACE2 secretase activity (ApoE$^{-/-}$ BACE2$^{-/-}$). In the aorta of these mice and of control ApoE$^{-/-}$ mice atherosclerotic lesions, represented by lipid-rich area, were visualized in red after oil red O-staining (**Figure 14a**). As shown in **Figure 14b** inactivation of BACE2 secretase activity had no effect on lesion formation neither in the thoracic (21.4±1.6% ApoE$^{-/-}$ vs. 20.5±2.3% ApoE$^{-/-}$ BACE2$^{-/-}$, n.s) nor in the abdominal aorta (27.3±2.3% ApoE$^{-/-}$ vs. 23.8±2.1% ApoE$^{-/-}$ BACE2$^{-/-}$, n.s).

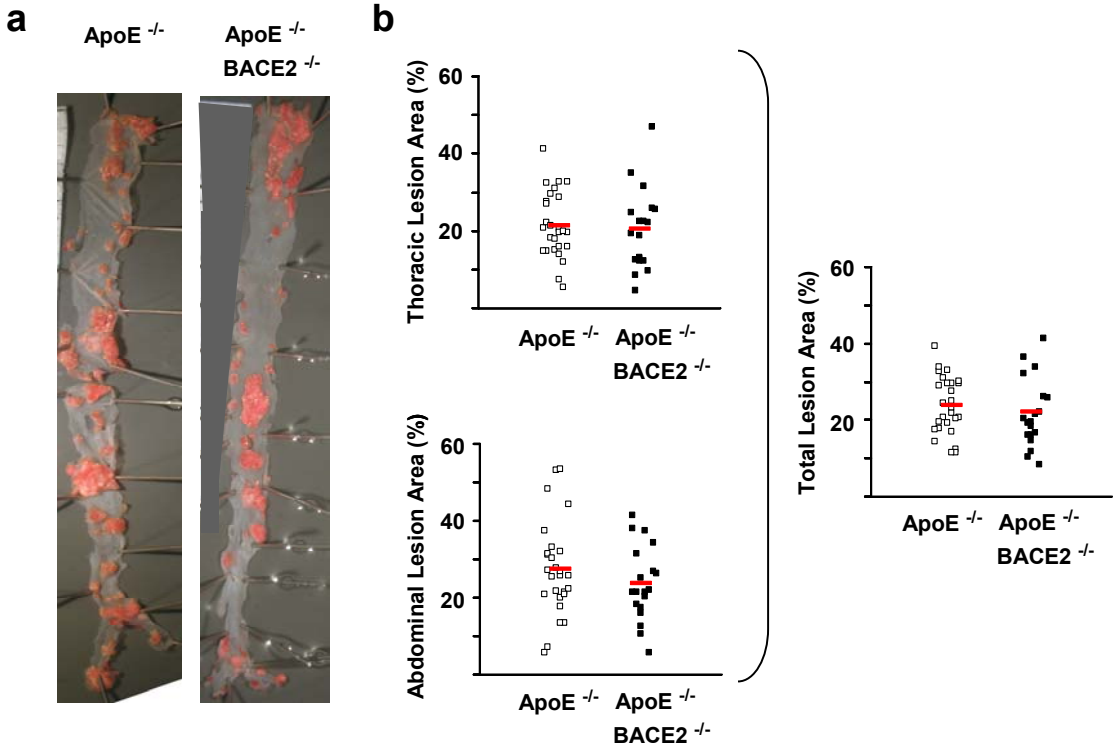


Figure 14: Effect of BACE2 secretase inactivation on atherosclerosis development in the aorta of ApoE$^{-/-}$ mice after high-fat/cholesterol diet.

a. Representative images of atherosclerotic plaques from longitudinally opened aortas (en face analysis) using oil red O staining in ApoE$^{-/-}$ and ApoE$^{-/-}$ BACE2$^{-/-}$ mice after high fat/cholesterol diet.

b. Quantification of atherosclerotic lesion area in total, thoracic and abdominal aorta in ApoE$^{-/-}$ and ApoE$^{-/-}$ BACE2$^{-/-}$ mice. Data represent means±SEM and are expressed as percentage of thoracic, abdominal, or total aorta, respectively. ApoE$^{-/-}$ (n=27) and ApoE$^{-/-}$ BACE2$^{-/-}$ (n=20).

Inactivation of BACE2 Secretase Activity Does Not Affect the Level of Pro-Inflammatory Cytokines, Lipids and Cholesterol in the Plasma of ApoE$^{-/-}$ Mice

The level of pro-inflammatory cytokines in the plasma of ApoE$^{-/-}$ BACE2$^{-/-}$ and control ApoE$^{-/-}$ mice were analysed. IL-1β, IL-6 and TNF-α were detected in the plasma of both animal groups. IL-6 was 2-fold higher expressed (at about 40 pg/ml, **Figure 15a**) compared with IL-1β and TNF-α, which were expressed at around 20 pg/ml (**Figure 15a**). No difference in cytokine levels were observed between groups.

The level of cholesterol, triglycerides and free fatty acids were also analysed. The concentration of total cholesterol in the plasma of these mice was at about 525 mg/dl, which is 7-fold higher compared with the triglycerides concentration. Free fatty acids level was ranging from 580 to 600 μM. No difference in cholesterol, triglyceride and free fatty acid levels between ApoE$^{-/-}$ BACE2$^{-/-}$ and control ApoE$^{-/-}$ mice were observed (**Figure 15b**).

The lipoprotein profile of both animal groups was analysed more in detail by FPLC analysis. The level of VLDL was producing the higher pick, reaching a concentration of about 0.65 mg/dl. LDL was 2-fold less concentrated (0.3 mg/dl) compared with VLDL. The level of the anti-atherogenic HDL was almost undetectable in both animal groups. No difference in lipoproteins was detected between ApoE$^{-/-}$ BACE2$^{-/-}$ and control mice using FPLC analysis (**Figure 15c**).

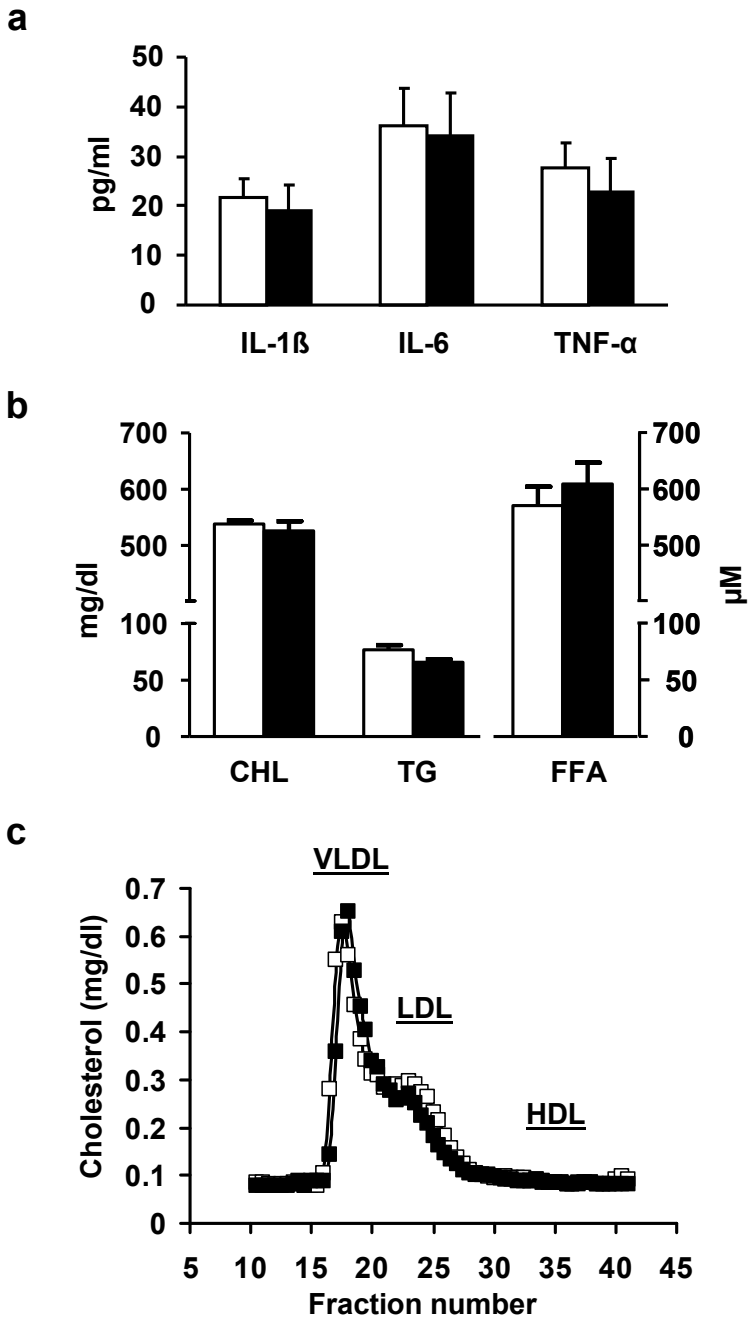


Figure 15: Effect of BACE2 secretase inactivation on plasma levels of pro-inflammatory cytokines, cholesterol and lipids after high-fat/cholesterol diet.

a. Plasma levels of IL-1β, IL-6 and TNF-α inflammatory cytokines were quantified in fasted ApoE$^{-/-}$ (open bars) and ApoE$^{-/-}$ BACE2$^{-/-}$ (filled bars) mice. Data represent means±SEM. n=9 per group.

b. Cholesterol (CHL), triglycerides (TG) and free fatty acids (FFA) were quantified in the plasma of fasted ApoE$^{-/-}$ (open bars) and ApoE$^{-/-}$ BACE2$^{-/-}$ (filled bars) mice. Data represent means±SEM. n=10 per group.

c. Lipoprotein profiles measured by FPLC analysis in fasted ApoE$^{-/-}$ (open symbols) and ApoE$^{-/-}$ BACE2$^{-/-}$ (filled symbols) mice. Data represent lipoprotein profile of pooled plasma from 5 animals.

Effect of BACE2 Secretase Inactivation on Metabolic Parameters in ApoE$^{-/-}$ Mice

Body weight of ApoE$^{-/-}$ BACE2$^{-/-}$ and control ApoE$^{-/-}$ mice was monitored. Prior to sacrifice ApoE$^{-/-}$ BACE2$^{-/-}$ mice showed 5% higher body weight compared with ApoE$^{-/-}$ mice (30.4±0.31 vs. 28.9±0.4, *P*=0.013, **Figure 16a**).

Glucose tolerance test was also performed in these mice. Mice with inactivated BACE2 catalytic activity had a better glucose tolerance as represented in **Figure 16b (**AUC: 1288.7±52.4 vs. 1732.7±59.4, *P*<0.0001). The insulin level in the plasma of both animal groups was ranging between 0.3 and 0.35 ng/ml, but no significant difference was observed between ApoE$^{-/-}$ BACE2$^{-/-}$ and control ApoE$^{-/-}$ mice (**Figure 16c**).

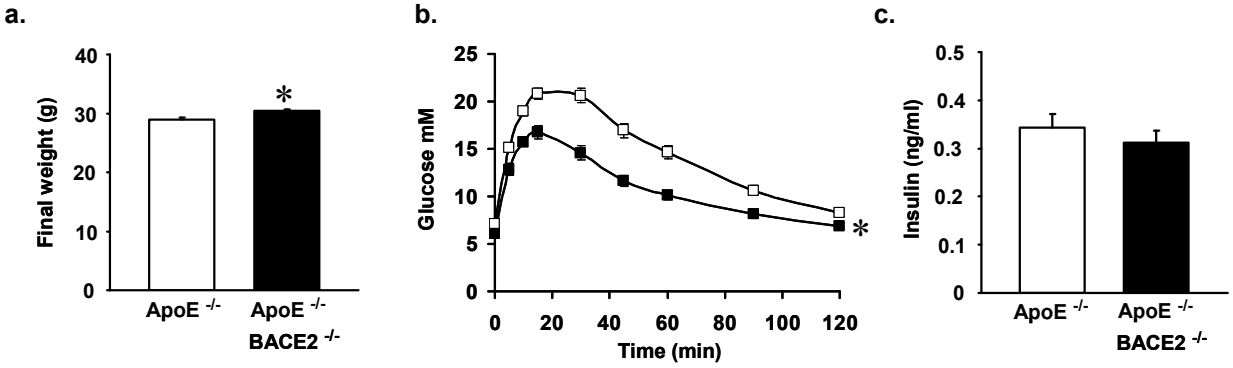


Figure 16: Effect of BACE2 secretase inactivation on metabolic parameters in ApoE$^{-/-}$ mice after high-fat/cholesterol diet.

a. Body weight was measured prior to sacrifice in fasted ApoE$^{-/-}$ (open bars) and ApoE$^{-/-}$ BACE2$^{-/-}$ (filled bars) mice. Data represent means±SEM. ApoE$^{-/-}$ (n=28) and ApoE$^{-/-}$ BACE2$^{-/-}$ (n=19), **P*=0.013 vs. ApoE$^{-/-}$ mice.

b. Glucose tolerance tests were performed prior to sacrifice in fasted ApoE$^{-/-}$ (open symbols) and ApoE$^{-/-}$ BACE2$^{-/-}$ (filled symbols) mice. Data represent means±SEM. ApoE$^{-/-}$ (n=31) and ApoE$^{-/-}$ BACE2$^{-/-}$ (n=21), AUC **P*<0.0001 vs. ApoE$^{-/-}$ mice.

c. Insulin plasma level was quantified in fasted ApoE$^{-/-}$ (open bars) and ApoE$^{-/-}$ BACE2$^{-/-}$ (filled bars) mice. Data represent means±SEM. n=10 per group.

3.2.4 Discussion

In this part of the study the effect of BACE2 secretase inactivation on atherosclerotic plaque formation, plasma level of inflammatory cytokines and metabolic parameters were examined *in vivo*. To do that, we analyzed ApoE knock-out mice lacking one of the two aspartic catalytic residues indispensable for BACE2 secretase activity [21] ($ApoE^{-/-}$ $BACE2^{-/-}$) after feeding the animals with a high fat/cholesterol diet.

Inactivation of BACE2 secretase activity had no effect on atherosclerotic plaque formation, plasma concentration of pro-inflammatory cytokines and plasma levels of cholesterol and lipids such as triglycerides and free fatty acids. Body weight, on the contrary, was slightly increased in $ApoE^{-/-}$ $BACE2^{-/-}$ mice compared to control $ApoE^{-/-}$ mice. The small difference is probably not relevant for the present study but rather a consequence of experimental variability. Surprisingly, glucose tolerance was significantly improved in the $ApoE^{-/-}$ $BACE2^{-/-}$ compared to control mice, despite a similar fasting plasma insulin concentration. The ameliorated glucose tolerance in these mice led to speculate a putative role of BACE2 in glucose metabolism. It is well known that the sensitivity to the glucose-lowering action of insulin is very variable [22]. It may be possible that the absence of BACE2 activity in the $ApoE^{-/-}$ $BACE2^{-/-}$ mice improves the sensitivity to insulin. To be able to confirm this hypothesis, an insulin tolerance test should be performed. Binding of insulin to its receptor stimulates the uptake of glucose into the cells [23], and BACE2 may possibly interfere with insulin-mediated signalling pathways leading to inhibition of glucose uptake. Skeletal muscle and/or adipose tissue are the major organs involved in storage and metabolism of glucose [23]. For instance, shedding of the transmembrane protein TNF-α receptor 2 has been negatively correlated with insulin sensitivity [24], suggesting a possible mechanistic link to our result. Further studies on that direction should be done to test this hypothesis.

In the manuscript draft previously presented a decrease in atherosclerotic plaque formation and a reduction in plasma concentration of inflammatory cytokines, cholesterol and lipids in $ApoE^{-/-}$ mice overexpressing BACE2 was observed. Having established that BACE2 had a protective role in these mice, one may expect an aggravation of the overall condition in regard to atherosclerosis in mice lacking BACE2 secretase activity. Surprisingly, this was not observed in our *ex-vivo* experiments. It can not be excluded, that the apparent normal phenotype of $ApoE^{-/-}$ $BACE2^{-/-}$ mice is due to a compensatory mechanism and/or genetic redundancy. Another protein able to compensate the role of BACE2 may be present in these mice to balance the lack in secretase activity. This phenomenon was demonstrated in mice

lacking the gene for calcium transport, CaBP-9K [25]. These mice showed no distinct phenotype compared to wild-type suggesting the presence of compensatory genes. Given that BACE2 has a high homology with BACE1 [26], and that both proteins share frequently the same substrate [26, 27], BACE1 may be the best candidate to compensate for the absence of catalytic activity of BACE2. A partially compensatory role of BACE1 and BACE2 was already demonstrated by Dominguez and co-workers in a previous study [21]. To analyze a possible overlapping function of the two homologous proteases, this group generated mice with inactivated BACE1, BACE2 or both secretase activities. This study demonstrated a higher mortality rate in mice having BACE1 secretase inactivated. On the contrary, and in support to our results, mice lacking BACE2 activity showed an overall healthy phenotype. Interestingly, double knock-out mice, had an higher mortality rate than the monogenic secretase inactive mice, suggesting a compensatory mechanism between the two homologous genes [21].

Therefore, a compensatory role of BACE1 may be speculated in the present as well. To be able to demonstrate this hypothesis, ApoE$^{-/-}$ mice lacking both BACE1 and BACE2 secretase activities should be generated and analyzed for atherosclerosis development and for other metabolic parameters. Considering the fact that double knock-out mice showed a higher rate of mortality in the study of Dominguez *et al.* [21], the creation of the ApoE$^{-/-}$ BACE1$^{-/-}$ BACE2$^{-/-}$ mice may not be feasible or recommended.

Other secretases apart from BACE1 could also be considered as good candidates to compensate the activity of BACE2 in the ApoE$^{-/-}$ BACE2$^{-/-}$ mice. α-Secretases of the ADAM-family, such as ADAM-17 [28], ADAM-10 [29], ADAM-9 [30] and ADAM-19 [31] were for example shown to cleave APP in the non-amylogenic pathway similarly to BACE2 [32, 33].

3.3 References

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4. EXTENDED DISCUSSION

4.1 Regulation of BACE2 Expression

In the present thesis, a proteasome-dependent degradation of BACE2 upon mitogenic stimuli was observed. This is a commonly used regulatory mechanism to rapidly downregulate cellular proteins. Transmembrane proteins such as PDGF, GH and EGF receptors have also been shown to be downregulated by this pathway [1]. Proteins with antiproliferative function, such as tumor suppressor p53, were described to be downregulated by the proteasome in many tumor cells in order to initiate cell cycle [2]. Similarly it has been shown in the present study. Since the downregulation of BACE2 observed after mitogenic stimuli is still present after 48h, it is highly possible that the rapid degradation of BACE2 protein is followed by a further regulation at transcriptional level. This is supported by the data presented in the manuscript draft, which show a decrease in BACE2 expression at mRNA level as well.
A better characterization of BACE2 on transcriptional level may help to achieve a more complete understanding of its regulation in VSMCs. The nucleotide sequence of BACE2 promoter was analyzed by different groups and potential consensus sequences of transcription factors were identified [3-5]. However, the *in silico* data generated should be further analyzed *in vitro* to identify DNA-binding proteins (transcription factors), which may possibly interact with the BACE2 promoter or enhancers regions. This could be investigated by performing assays such as electrophoretic mobility shift (EMSA), DNase I footprinting assay and chromatin immunoprecipitation assay (ChIP) [6]. The transcription factors identified using this approaches may be validated in VSMCs.

4.2 BACE2, an Alternative α-Secretase: Role of α-Secretases in Atherosclerosis

As described in the introduction of this dissertation, α-secretases [7] including BACE2 [8, 9] prevent Alzheimer's disease by participating in the non-amylogenic processing of APP. BACE2 in fact was described to preferentially cleave APP between phenylalanine 190 and phenylalanine 191, which is 3 amino acids after the α-secretase cleavage site [9]. Enzymes of the ADAM family belong to the category of the α-secretases. Dependent on the cell type,

ADAM proteins have a variety of physiological functions, including regulation of cell growth, migration, signalling and homeostasis [10]. Not surprisingly, these proteins, being involved in so many processes, are also linked to different diseases such as cancer, Alzheimer's disease and cardiovascular diseases [10]. During atherosclerosis, ADAM proteins are shown to be importantly involved in the inflammatory processes by inducing ectodomain shedding of different transmembrane proteins [10]. In leukocytes, metalloprotease-dependent shedding of L-selectin, is known to inhibit leukocytes adherence to the endothelial cells [11], thereby preventing rolling and emigration of these cells into the arterial wall. Shedding of ICAM-1 and V-CAM-1 by ADAM-17[12], on the contrary, is promoting leukocyte-rolling. VE-cadherin shedding by ADAM-10 [13] enhances vascular permeability for leukocytes, by disturbing intracellular junction of endothelial cells [12]. ADAM-17 also mediates extracellular shedding of cytokines such as pro-TNF-α, regulating inflammatory responses during atherogenesis [14]. Moreover, metalloproteaseas such as ADAMDEC1 are also involved in plaque destabilisation by proteolitically cleaving the extracellular matrix of an advanced fibrous lesion [14]. Notch signalling activation triggered by sequential ADAM and γ-secretase cleavage resulting in the released of an intracellular domain, is known to activate macrophages, which then express high amounts of inflammatory molecules [15].

It is evident that α-secretases are involved in many biological processes, and that their function is much dependent from the molecular context of their expression. The same should also be considered for BACE2. The role of this aspartyl protease observed in VSMCs in this study, may in fact differ significantly at different stage of atherosclerosis or in other cell types.

4.3 Possible Role of BACE2 in Cytokine Receptor Shedding

In the present study a reduced activation of AP-1- and NF-κB-dependent pro-inflammatory signaling pathways in VSMCs overexpressing BACE2 were observed. These transcription factors are frequently activated via binding of pro-inflammatory cytokines to their cognate receptors [16, 17].

Interestingly, the IL-1 receptor type II (IL-1R2), a decoy receptor homologous to IL-1RI transmembrane receptor, has been identified as a substrate of BACE2 [18]. Recently, Elzinga and co workers demonstrated that IL-1R1 undergoes proteolytic processing [19]. Binding of IL-1β to IL-1R1 mediates activation of both NF-κB and AP-1 transcription factors [20] and, importantly, this cytokine receptor is known to be expressed in VSMCs [21] and also to be

localized in lipid rafts [22], as shown in this thesis for BACE2. A prior study suggests α- β- and γ-secretases-mediated cleavage to occur preferentially in these membrane compartments [23]. The possible BACE2-mediated shedding of IL-1R1 and IL-1R2 may increase the availability of decoy receptors in the blood plasma and lead to a loss of receptor function. These mechanisms may be responsible for the downregulation of intracellular c-jun- and IκBα-dependent pro-inflammatory and mitogenic signaling cascade. **Figure 17** depicts a schematic representation of the putative role by which BACE2 mediates downregulation of cytokine receptor signaling.

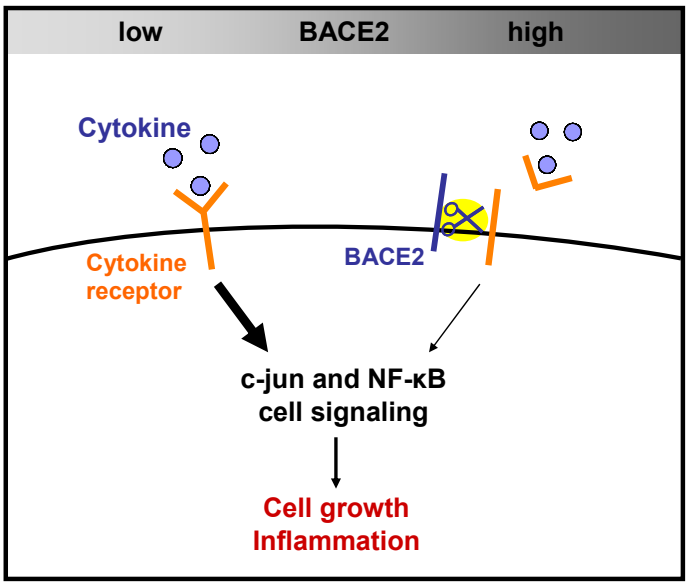

Figure 17: Model for BACE2-dependent downregulation of pro-inflammatory pathways and proliferation. Shedding of the extracellular part of a cytokine receptor by BACE2 may result in downregulation of downstream pro-inflammatory signaling pathways, inhibiting the transcription of inflammatory and mitogenic molecules.

The speculation that IL-1R1 and/or Il-1R2 decapitation is/are involved in the anti-atherogenic action of BACE2 is supported by the observation that IL-1 induces expression of adhesion molecules in endothelial cells [24] and promotes proliferation in VSMCs [25], underscoring the importance of IL-1 during atherogenesis [25]. However, is very likely that other receptor/s involved in inflammation and proliferation in addition to IL-1R1 is/are cleaved by BACE2. Therefore, an unbiased substrate screening approach of the entire cell proteome may help to identify BACE2 substrates responsible for the phenotype observed in VSMCs.

Today, the development of tools to compare proteomes quantitatively enables the identification of differences occurring from genetic or treatment disparity [26]. In a recent study

Hemming and co-workers identified γ-secretase substrates by metabolically labeling cellular amino acids with heavy or light isotopes [27]. Subsequently, the membrane proteins from control cells and cells treated with a γ-secretase inhibitor were separated using SDS-PAGE and analyzed by mass spectrometry, which differentiates between heavy and light isotopes.
A possible approach to identify putative substrates of BACE2 responsible for the anti-atherogenic properties observed in our study could be to perform a general substrate screening using control and BACE2 overexpressing VSMCs. A comparative proteomic analysis of the culture supernatant of control and VSMCs overexpressing BACE2 may also give important evidence for shedded products. This approach was used to identify new substrates of the metalloprotease ADAM17 [28], where conditioned medium derived from ADAM17 overexpressing cells treated with or without a metalloprotease inhibitor was subjected to agarose chromatography and the purified glycoproteins were labeled with Cy3 or Cy5 and analyzed by 2D gel electrophoresis. Spots differing in intensity were excised and processed by ionization mass spectroscopy to identify proteins. Alternatively, instead of analyzing shedded proteins present in the cell supernatant, the membrane fraction of the cells could be analyzed by mass spectrometry, to identify extracellular protein domains which are missing in cells overexpressing BACE2. A similar approach was used by Lund and co-workers to identify metastasis-associated cell surface markers [29]. In this study surface membrane proteins of metastatic and non-metastatic cell line were isolated and separated by density gradient, followed by a dual stable isotopic labeling by amino acids (SILAC) of the proteomes. Mass spectrometry analysis was used to identify and quantify proteins that were differentially expressed. The identification of a potential substrate of BACE2 in VSMCs, which may explain the mechanism involved in the anti-atherogenic action of the secretase, may give important information to mechanistically explain the results described in the present thesis.

4.4 *In vivo* relevance?

To investigate whether the effect of BACE2 observed in VSMCs has *in vivo* relevance, the effect of overexpression and secretase inactivation of BACE2 was analyzed in a well established mouse model of atherosclerosis: the apolipoprotein E knock-out mouse ($ApoE^{-/-}$). These mice develop spontaneously hypercholesterolemia and atherosclerotic plaques [30, 31]. For the *in vivo* experiments performed in this study, only male mice were used, as estrogen was shown to reduce lesion formation in $ApoE^{-/-}$ mice [32].

The transgenic mice generated in this study overexpress human BACE2 protein. Importantly, the amino acid sequence of human and murine BACE2 shares 88% identities, whereby the two catalytic regions are completely orthologous as verified by blast analysis. Therefore it appears highly likely that human and murine BACE2 proteins have highly similar substrate recognition sites.

In the *in vivo* experiments we demonstrated a reduction in atherosclerotic plaque area and in plasma concentration of pro-inflammatory cytokine IL-1β in $ApoE^{-/-}$ mice overexpressing BACE2 ($ApoE^{-/-}$ BACE2-Tg). Surprisingly, the opposite effect was not observed in mice lacking BACE2 secretase activity ($ApoE^{-/-}$ $BACE2^{-/-}$). As already discussed (see: discussion part of the *in vivo* experiments not included in the manuscript draft), a compensatory mechanism may be involved. It is important to consider that the choice of the duration of the high fat/cholesterol diet treatment may influence considerably the *in vivo* results. The atherosclerotic plaques that were observed in $ApoE^{-/-}$, $ApoE^{-/-}$ BACE2-Tg and $ApoE^{-/-}$ $BACE2^{-/-}$ mice after 15 weeks of high fat/cholesterol diet treatment are already at an advanced stage. Performing the *in vivo* experiments at an earlier time point, may allow to delineate differences between mice strains.

Importantly, the results generated *in vivo* are compatible with the *in vitro* observations and with the hypothesis of IL-1R1 and IL-1R2 decapitation. In fact, activation of this cytokine receptor is known to amplify the inflammatory response by expressing different cytokines, including IL-1 itself. This may explain the reduced concentration of IL-1 in the plasma of BACE2-Tg mice. Considering that the transgenic mice generated in this study overexpress BACE2 ubiquitously and that IL-1 and IL-1R1 are produced by different cells involved in the pathogenesis of atherosclerosis [33], the involvement of other cells other than VSMCs in the anti-atherogenic effect of BACE2 is highly likely.

It would be interesting to generate mice overexpressing BACE2 specifically in VSMCs. The data obtained using these mice may help to delineate which changes observed in BACE2-Tg mice compared to control mice are an effect of increased expression of BACE2 in VSMCs and which effects are mediated by other cells types. A mammalian expression vector coding for BACE2 under the control of a smooth muscle cell specific promoter (SM22-promoter) was created, however we were not able to generate a transgenic founder after nuclear injection of oocytes.

It is important to consider that other cells beside VSMCs are indispensable for the development of an atherosclerotic lesion. Macrophages and lymphocytes are also of crucial importance during atherogenesis. These cells extravasate from the blood into the

subendothelial space, where they proliferate and contribute to the local chronic inflammation by releasing huge amount of cytokines [34]. Definitely, it would be interesting to find out if the effects of BACE2 overexpression that we observed in VSMCs, may be reproduced also in macrophages or lymphocytes. BACE2 may in fact have a proliferation/inflammation-regulatory role in other vascular cells in addition to VSMCs. To examine the role of macrophages and lymphocytes in the BACE2 transgenic mice, a bone marrow transplantation experiment may be performed. Bone marrow is the main hematopoietic organ which is responsible for the production of blood cells, including macrophages and lymphocytes [35]. Bone marrow from ApoE$^{-/-}$ BACE2-Tg mice may be transplanted into control ApoE$^{-/-}$ mice followed by atherosclerotic plaque quantification. If the recipient mice show a decreased formation of atherosclerotic lesions, after bone marrow transplantation, this may indicate that bone marrow-derived cells (such as macrophages and lymphocytes) may be involved in the BACE2-dependent decrease in atherosclerosis development. This approach was used by Dan Yang and co-workers to investigate the role of adenosine receptor in VSMCs and bone marrow in regard to vascular injury [36].

Additionally, since expression of the mutated BACE2 gene lacking secretase activity was analyzed in this study only *in vitro*, an interesting approach could be the generation of ApoE knock-out mice overexpressing BACE2 lacking secretase activity. These mice may in fact confirm *in vivo* the secretase-dependent effect of BACE2 on atherosclerosis development. VSMCs from these mice, BACE2 transgenic mice and ApoE$^{-/-}$ control mice could also be explanted and cultured to further perform *in vitro* experiments to directly link our *in vitro* and *in vivo* results.

4.5 Possible Roles of BACE2 in Regulation of Lipid and Cholesterol Metabolism

The decrease of the inflammatory cytokine IL1-β in the plasma of ApoE$^{-/-}$ BACE2 transgenic mice, was associated with lower cholesterol, triglycerides and free fatty acid concentrations, suggesting a possible role of BACE2 in the regulation of lipid and cholesterol metabolism.

Lipids and cholesterol are insoluble in water; therefore, in the plasma these molecules are transported in lipoproteins such as VLDL, LDL and HDL [37]. VLDL are responsible for the transport of triglycerides and cholesterol from the liver into the blood stream, where they are further catabolized to LDL. LDL molecules carry cholesterol and lipids to peripheral tissues

and in part to the liver. HDL have an important role in the transport of cholesterol back to the liver, where it may be excreted in form of bile acid [38, 39]. Lipoprotein receptors play an indispensable role for cholesterol and lipids homeostasis by clearing these molecules from the blood by receptor-mediated endocytosis [38]. Therefore, the transcriptional or post-translational regulation of receptors responsible of lipoprotein clearance is highly important. The LDL receptor family is composed of seven related transmembrane proteins [40]. One of these receptors, the low-density lipoprotein receptor related protein (LRP1), is of special interest. LRP1 is known to be widely expressed in the body, but particularly abundant in VSMCs, hepatocytes and neurons [40]. Moreover, LRP1 is known to undergo shedding [41]. The cleavage of LRP1 is shown to occur through the action of BACE1 in neurons [42], and through the action of metalloproteases in hepatocytes [43]. The biological significance of this ectodomain shedding is still unknown. Because BACE2 is known to cleave transmembrane protein similarly to α-secretases metalloproteases [8, 44], and to share often the same substrate with BACE1 [18], one could speculate a putative role of BACE2 in the cleavage of LRP1 in ApoE$^{-/-}$ BACE2-Tg mice, releasing a soluble receptor into the plasma. Moreover, in addition to lipoprotein, LRP1 is known to bind to different ligands, such as cytokines and growth factors [45]. A putative binding of the LRP1 soluble form (sLRP1) to pro-inflammatory cytokines present in the blood may therefore also be speculated. By this mechanism ("decoy receptor") sLRP1 may competitively inhibit binding of inflammatory cytokines to cell-surface-bound cytokine receptors. The formation of complexes containing either sLRP1 and cytokine or sLRP1 and LDL may increase the clearance of these complexes from the plasma. Similarly, an involvement of LRP1 in the clearance of Aβ from the blood stream through a potential transport of Aβ in the liver was recently speculated [46]. This hypothesis may explain the decreased inflammation, hypercholesterolemia and hyperlipidemia observed in ApoE$^{-/-}$ BACE2-Tg mice. Further studies in that direction should be performed to test this hypothesis. Interestingly, it has been shown in a recent study that processing of LRP1 limits inflammatory response in vitro and in vivo [47]. A proteomic analysis comparing plasma of transgenic versus control mice may help to identify a possible substrate released into the blood circulation through the action of BACE2. Zhang and co-workers demonstrated that tissue-derived proteins are present and detectable in the plasma, using mass spectrometry analysis of glycopeptides [48]. This technique may give the possibility to discover plasma protein biomarker related to diseases.

Lipid and cholesterol metabolism are complexly regulated on transcriptional or posttranscriptional level [49, 50]. To monitor the amount of membrane cholesterol, cells mainly

use transmembrane proteins with a sterol-sensing domain in the intramembrane sequence [49]. These proteins, including Scap and HMG CoA reductase, can induce or block the de-novo synthesis of cholesterol by monitoring the concentration of sterol inside the cell. The de-novo synthesis of cholesterol is mediated by 20 different enzymes, all of which are regulated by SREBP transcription factor family [49]. The synthesis of trigycerides and free fatty acids is also transcriptionally regulated and SREBP transcription factors are also known to be involved [50].
Interestingly, and in support to our results, multiple cytokines, including IL-1, are described to induce lipolysis, resulting in an increased secretion of VLDL, thereby raising the levels of triglycerides, free fatty acids and cholesterol in the plasma. This may therefore explain the reduction in lipid and cholesterol plasma concentration observed in BACE2-Tg mice in response to the drastic decrease of IL-1 levels in the circulation.
Being the cholesterol and lipid metabolism so highly complex, until further analysis are performed no conclusion can be drawn. Regulation at different levels appears likely as the changes observed in lipid and cholesterol concentration in the BACE2-Tg mice compared to control mice were dramatic.

4.5 Conclusion and Clinical Implications

In the present study, I showed for the first time that overexpression of BACE2 results in inhibition of cell proliferation and attenuation of pro-inflammatory signal cascades in VSMCs. On the other hand, BACE2 downregulation increased cell growth. In atherogenic mice, BACE2 was involved in inhibiting plaque formation, lowering plasma concentration levels of inflammatory cytokine IL-1β, cholesterol and lipids. Collectively, these data suggest a protective role of BACE2 in atherosclerosis development. For this reason, an attractive therapeutic approach would be to increase expression of this secretase in patients suffering from atherosclerosis.
A strategy to achieve an increased BACE2 expression would be to block its proteasomal degradation. This could be achieved by reducing the proteasomal activity, which have been shown to be overstimulated in atherosclerosis [51]. The development of drugs which inhibit protein degradation through proteasomal pathways is an intensive field of research in cancer treatment, where a dysregulated protein degradation is often occurring [52, 53]. The discovery of a specific drug which specifically inhibits BACE2 proteasomal degradation may provide a successful therapeutic approach to decrease progression of atherosclerosis lesions or to even prevent their formation.

Gene therapy represents an additional new attractive approach in vascular medicine. Adenovirus-mediated fibromodulin gene transfer has been previously performed in surgically prepared human saphenous veins [54]. Similarly, it could be possible to transduce BACE2 gene in vessels to be used for grafting, which may protect the grafted vessels from future lesion formation.

Together, the present data give new insight of a protein, commonly perceived to be important in Alzheimer's disease, as a candidate for development new therapies in atherosclerosis.

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